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The Hippo Signaling Pathway and Cancer

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ISBN 978-1-4614-6219-4 ISBN 978-1-4614-6220-0 (eBook)
DOI 10.1007/978-1-4614-6220-0
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013931949

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Contents

Chapter 1
Introduction

Moshe Oren and Yael Aylon

Keywords Hippo Pathway • Lats • Hpo • Yap

Twenty years ago marks a fascinating beginning of new fledgling life of a novel signaling cascade—the Hippo Pathway. Today we are still grasping to understand its biological context and scrabbling to find new modulators of the pathway. Research is in full swing, and does not show signs of slowing down in the foreseeable future.

The founding member of the Hippo pathway was Yap (YES-associated protein), first described in 1994 (Sudol 1994). As its name implies, Yap cDNA was isolated from a lambda phage expression library in a screen for proteins that bind to the Yes kinase (Sudol 1994). Sequence comparison between Yap proteins of different organisms revealed a new protein module; the WW domain. Subsequently, using a cDNA expression library, the WW domain of Yap was found to bind proline-rich peptides (Sudol et al. 1995). These motifs were to become linchpins of Hippo interactions.

Key to the ensuing discovery of additional Hippo components has been the rewarding exchange between mammalian and fly researchers (fruitful fruit flies!). Mosaic screens in *Drosophila* have facilitated the isolation of hyperproliferation mutations that are lethal at earlier developmental stages, since clusters of somatic cells mutated in genes that encode negative regulators of cell proliferation were easily detected. By 1995, more than 22 putative fly "tumor suppressor" genes had been cloned and characterized at the molecular level (Watson et al. 1994), four of which functioned in imaginal discs. These four were homologous to human genes: ***fat***, a gene encoding a large cadherin-like transmembrane molecule involved in cell adhesion (Mahoney et al. 1991); discs-large (***dlg***), encoding an SH3-containing kinase localized to cell junctions (Woods and Bryant 1991); lethal2giant larvae

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_1,

(***lgl***), localized either in the cytoplasm or in association with cell membranes at sites of cell–cell contact (Strand et al. 1994); and expanded (***exp***), the homolog of the mammalian NF2 tumor suppressor gene (Boedigheimer et al. 1993), which encodes a membrane-cytoskeleton linker protein. This is interesting not only because loss of apical–basal polarity and cell contact inhibition are critical attributes driving epithelial tumor progression but also because each of the proteins encoded by these genes was ultimately implicated in signaling to the Hippo pathway (Grusche et al. 2010).

dLats (Large tumor suppressor or warts, ***wts***) is an archetypal example of a hippo-related tumor suppressor isolated in a *Drosophila* screen. ***dLats*** was identified in 1995 in two independent screens for recessive hyperproliferation mutations (Justice et al. 1995; Xu et al. 1995). It was recognized as a member of the NDR family of kinases and was subsequently the first ser/thr kinase demonstrated to negatively regulate cell cycle (Xu et al. 1995). Loss of ***dLats*** caused a massive hyperproliferation phenotype with enlarged adult somatic structures (Xu et al. 1995). Mutant flies also exhibited apical hypertrophy of epithelial cells, leading to abnormal deposition of extracellular matrix during adult development ("warts") (Justice et al. 1995). Proline-rich regions were identified in the N-terminus of the protein. In retrospect, this is evocative of the PP domain that had been highlighted previously by Sudol within the Yap-binding context (Sudol 1994).

Mammals harbor two ***dLats*** orthologs, Lats1 and Lats2. Mice lacking Lats1 develop soft-tissue sarcomas and ovarian stromal tumors with 100% penetrance, and are highly sensitive to exposure to carcinogens (St John et al. 1999). This was an important milestone, since it indisputably pegged Lats as a tumor suppressor.

In 2000, Taz (Transcriptional co-activator with PDZ-binding motif) was isolated in a cDNA screen for novel 14-3-3-binding proteins (Kanai et al. 2000). BLAST analysis revealed significant sequence homology and domain conservation with the Hippo component Yap (Both Yap and Taz contain WW domains, although Yap does not harbor a PDZ domain and Taz does not harbor a Yes-binding SH3 domain). The transcriptional co-activation function of Taz was dependent on its C-terminal PDZ domain. This was intriguing, since PDZ domains historically have been found in membrane-associated signal transduction molecules, such as the tight junction protein ZO1. Consistent with this, Taz could be found at the plasma membrane, as well as in punctate nuclear foci. The authors foresaw that competition between PDZ domain-mediated membrane and nuclear targeting, along with phosphorylation-dependent 14-3-3 binding and cytoplasmic sequestration, might provide a mechanism for spatial control of Taz function (Kanai et al. 2000).

Meanwhile, work on dissecting the transcriptional function of Yap bustled on. Yap was shown to function as a coactivator for a number of transcription factors, such as the Runx family member PEBP2a (Yagi et al. 1999) and p73 (Strano et al. 2001). Runx family members play an important role in regulating mesenchymal stem cell differentiation during bone formation (Lian et al. 2004). Yap and p73, a well-known member of the p53 family, act together in a feed-forward circuit to drive apoptosis (Basu et al. 2003; Lapi et al. 2008; Levy et al. 2007). The above interactions were mediated by the WW domains of Yap and the PPxY motifs of PEBP2a and p73. More recent work has highlighted the direct interaction of Yap and Taz with the four

TEAD/TEF family transcription factors, which mediate Yap- and Taz-dependent tissue growth and progenitor cell expansion (Vassilev et al. 2001). Interestingly, this binding was independent of the Yap and Taz WW domains (Chen et al. 2010; Li et al. 2010; Tian et al. 2010).

In another *Drosophila* screen, akin to that of Xu et al. described above, Tapon (2002) identified mutations in Salvador (***sav***, named after the surrealist painter Salvador Dali who, while alive, claimed to be immortal). Concurrently, in a genetic screen to identify mutations that affected *Drosophila* eye size, the group of Georg Halder isolated the same gene and called it *shar-pei* (Kango-Singh et al. 2002). ***Sav*** protein contained two WW domains that were necessary for its interaction with ***dLats*** (Tapon et al. 2002). Similar to ***dLats***, ***sav*** mutant cells proliferated more than their wild-type counterparts. Although tissue patterning appeared unaffected, an excess of a subtype of cells whose number was normally "pruned" by apoptosis was suggestive of a defect in cell death. These similarities, as well as a physical interaction, led the authors to postulate that ***sav*** and ***dLats*** may work epistatically. Complicating this possibility, however, was the fact that double mutants exhibited more severe phenotypes than either single mutant, suggesting that the two genes did not work in a simple linear manner.

In 2003, hippo (***hpo***), a ser/thr kinase and ortholog of Mst1/2, was identified by no less than five independent groups: four by genetic mosaic screens (similar to those described above) for mutants exhibiting hyperproliferation (Udan et al. 2003; Wu et al. 2003; Harvey et al. 2003; Jia et al. 2003) and one in a yeast two hybrid screen using ***sav*** as bait (Pantalacci et al. 2003). Reminiscent of ***dLats*** and ***sav***, ***hpo*** mutants displayed high levels of cyclin E which drives cell proliferation, as well as increased ***dIAP1***, an inhibitor of apoptosis. ***Hpo*** physically bound ***sav***, which in turn interacted with ***dLats***, suggesting that the three proteins functioned as a complex to negatively regulate cell proliferation. The trio was postulated to act via transcriptional repression of cyclin E and dIAP1, by unknown mechanisms. This new "Hippo" complex had only a handful of identified kinase substrates; a destabilizing phosphorylation of dIAP1 (Tapon et al. 2002; Harvey et al. 2003; Pantalacci et al. 2003), the G2/M regulator cdc2 (Tapon et al. 2002; Tao et al. 1999) and actin regulators zyxin and LIMK1 (Hirota et al. 2000; Yang et al. 2004). Furthermore, ***sav*** was shown to be a target of ***hpo*** kinase, and ***sav*** and ***hpo***, jointly, promoted phosphorylation of ***dLats*** (Wu et al. 2003; Pantalacci et al. 2003; Chan et al. 2005). Although the "Hippo" complex clearly affected transcriptional levels of cyclin E and dIAP1, nothing was known about how the pertinent signals were transduced into the nucleus and/or integrated with other transcriptional programs.

In 2005 the pieces started to come together. Implementing a yeast two hybrid screen using the N-terminus of ***dLats*** as bait, Duojia Pan's group (Huang et al. 2005) identified yorkie (***yki***), the fly ortholog of Yap, as a critical target of the Lats kinase. Accordingly, ***yki*** was required for dIAP1 transcription, whereas overexpression of ***yki*** phenocopied loss-of-function mutations of ***hpo***, ***sav***, and ***dLats*** (Huang et al. 2005). Thus, ***yki*** was the first substrate identified for the Hippo pathway, and, more broadly, for any of the NDR kinases. The authors also noted that NDR kinases are often regulated by a family of proteins called Mob. Congruently, in *Drosophila*, the

Mob family protein ***Mats*** was identified as a tumor suppressor putatively regulating Lats in the Hippo signaling pathway (Lai et al. 2005).

Subsequent work reinforced the notion that the "canonical" mechanism of Hippo regulation is via cell–cell contact. In tissue culture, high cell density induced phosphorylation, cytoplasmic translocation, (Zhao et al. 2007) and rapid degradation (Zhao et al. 2010) of Yap. Accordingly, disruption of cell junctions in epithelial cells resulted in the nuclear localization of both Yap and Taz (Varelas et al. 2010).

Thus, the Hippo signaling pathway was born—but where are we now?

The study of Hippo function continues to be enormously exciting and persistently surprising. The authors of the chapters in this book are at the cutting edge of the Hippo field. We will allow their chapters to speak for themselves.

Lest we be lulled into the complacent opinion that the Hippo pathway has been "deciphered," let us remember that while a coherent conception of Hippo functioning is now emerging, additional evidence of more complex networks of interactions is also discernible. Illustrating just one of many examples, even at the time of placing Mst as the central Hippo kinase, data had already accumulated of seemingly Hippo-unrelated functions of Mst kinases. Mst1 and 2 had been described as MAPKKK kinases that incite c-Jun, p38 and caspase activation (Graves et al. 1998). Once activated, caspase 6/7 cleaves Mst1 (whereas caspase 3 cleaves Mst2) (Feig and Buchsbaum 2002), creating a constitutively activated kinase that is transported into the nucleus to phosphorylate histone H2B and potentiate apoptotic chromatin condensation (Cheung et al. 2003). The new Hippo pathway added another level of complexity to this preexisting story. The cleaved portion of Mst harbors a SARAH (SAlvador-RAssf-Hpo binding) motif, which keeps the Mst pro-apoptotic function in check. In response to oncogene activation, for instance, the Hippo components ***sav*** and Rassf1a displace inhibitory Raf1, thereby activating an apoptotic Mst-Lats kinase signaling cascade (O'Neill and Kolch 2005).

Evidence for the involvement of subpopulations of Hippo components in non-Yap/Taz effector outcomes continues to crop up. Most of these processes have been less "neatly" resolved than the above Mst story. In fact, the first description of an in vivo upstream activating signal (in this case, DNA damage) of the Hippo pathway actually involved dmp53, the fly ortholog of the p53 tumor suppressor (Colombani et al. 2006). Similarly to its mammalian counterpart, dmp53 mediates the DNA damage response in the fly. Importantly, ***Hpo*** signaling is required for a maximal dmp53 response. In turn, ***Hpo*** kinase activity is activated in a dmp53-dependent manner (Colombani et al. 2006). Concurrently, our laboratory uncovered a somewhat analogous feedback circuit in mammalian cells (Aylon et al. 2006). In the mammalian system, mitotic or oncogenic stress causes Lats2 to translocate from the centrosome to the nucleus. In the nucleus, Lats2 binds the negative regulator of p53, Mdm2, leading to inhibition of p53 degradation and induction of a p53-driven transcriptional response. Since the Lats2 gene itself is directly transcriptionally activated by p53, this leads to a gradual and continuous increase in Lats2 protein levels.

The mention of p53 is not coincidental. p53 is historically one of the most studied tumor suppressor genes, making it the prototypic tumor suppressor. p53 was identified in 1979 by four independent laboratories (Lane and Crawford 1979;

Linzer and Levine 1979; DeLeo et al. 1979; Kress et al. 1979). Following its discovery, p53 has evolved from an obscure molecule to a key tumor suppressor gene with potentially great clinical impact. In many ways, the p53 pathway is long considered to have come of age. In this analogy, the Hippo pathway is still a toddler, but perhaps we can learn from the flip-flopped evolution of our concept of p53 tumor suppressor function and apply similar principles to the burgeoning Hippo pathway.

Three major factors have contributed to the overwhelming success of p53 research; (1) reliable working "tools" and infrastructure; (2) recognition of cross-talk with other pathways and (3) clinical relevance.

How do these attributes apply to the Hippo pathway? For tools, we need a battery of reliable and sensitive measuring and detection methods; good antibodies, strong mouse models, identification of a robust list of target genes; good database infrastructure to make information accessible and interchangeable to all researchers. We need to continue to meet at conferences, talk, discuss, exchange reagents, and ideas.

As to recognition of cross-talk with other pathways, our understanding of the intricacies of cell signaling begins on the single molecule level. Genetic and physical interactions develop into pathways, which subsequently evolve into cellular networks. But even networks do not function in a vacuum. Cell fate decisions are the sum total of innumerous signaling inputs and outputs, the weight of each signal being determined (among many other factors) by cell density, cell type, developmental stage, neighboring cells, and whether those cells are normal or transformed. Complicating the "untangling" of distinct networks is the fact that adult organisms often reuse signaling cassettes that were previously used for different purposes earlier in development. Furthermore, miswiring or hijacking of pathway members from diverse networks is often associated with severe diseases, such as cancer. From the Hippo perspective, different cells have distinct modifications of hippo function and output. One of the most glaring examples of this is the ability of Yap to promote tremendously diverse cellular outcomes such as apoptosis, cell growth, or "stemness." Whereas, on a broad level, this is suggestive of fail-safe mechanisms to check and limit the oncogenic potential of Yap-TEAD, it also implies a complex interaction between cellular signaling pathways.

As for clinical relevance, let us keep in mind that model organisms are just, well, model organisms. The exchange of information between *Drosophila* and mammalian systems has been very rewarding. However, care needs to be exercised against hasty analogies, since mammals are not merely wingless flies. Human genomics, such as identifying single nucleotide polymorphisms, copy number variations, and somatic mutations, are becoming more and more mainstream. Although confronting the genetic variations among humans is more "inconvenient" than working with inbred strains of model organisms, an immense advantage of humans is the detailed phenotypes that can be followed in clinical records. Comprehensive records of patient outcome, together with detailed genetic information, are rapidly being assembled in central facilities. Accessible and user-friendly databases will be critical for human-as-an-ultimate-model-organism Hippo researchers.

With this high-throughput vision in place, as well as the functions of the Hippo pathway expanding, and considering its central role in tumorigenesis and

development, the opportunities for drug development increase. Drugs that disrupt Yap-TEAD binding (Verteporfin), Taz-TEAD binding (TM-25659), or Yap nuclear translocation (Dobutamine) are already available (Jang et al. 2012; Liu-Chittenden et al. 2012; Bao et al. 2011). Conceivably, negative modulators of Hippo function are also potential drug targets. For instance, the PP2A phosphatase complex, an antagonist of ***Hpo*** (Ribeiro et al. 2010), is targeted by Fostriecin, which entered phase I clinical studies in 2002 as a cancer-killing agent (Lewy et al. 2002). Another exciting approach will be the search for targets in pathways that show synthetic lethality with either loss of Hippo function or excessive Yap/Taz function. Clinical applications are important not only because they save lives but also because they provide glimpses of the complex modes of action of molecules and pathways within a holistic human context.

We hope that by bringing together contributions from many leading experts, this volume will provide a great introduction to the field for newcomers to the Hippo pathway, as well as a starting point for vigorous debate among the already converted. The many unknowns in this system, detailed and discussed exquisitely in this volume, provide us all with inspiration for future work.

We are enormously indebted to the team of authors who took a timeout from their ongoing investigations to consider their work in a broader context and share it with us all, in true Hippo spirit!

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Part I
Up-stream Components of Hippo Signaling

Chapter 2
Merlin and Angiomotin in Hippo-Yap Signaling

Chunling Yi and Joseph Kissil

Abstract Merlin, encoded by the *NF2* tumor-suppressive gene, has been established through genetic studies in both *Drosophila* and mice as an important upstream regulator of the Hippo-Yap pathway. Recently, biochemical studies have identified Angiomotin and Angiomotin-like proteins as major interacting partners for both Merlin and Yap. The exact mechanisms of how Merlin and Angiomotin regulate Hippo signaling remain undetermined. In this chapter, we will summarize past findings and discuss controversies and remaining questions regarding the roles of Merlin and Angiomotin in Hippo signaling and tumorigenesis.

Keywords Merlin • NF2 • Adherens junctions • Tight junctions • Angiomotin • Hippo-Yap pathway

2.1 Neurofibromatosis Type 2 and Merlin

Neurofibromatosis type 2 is an inherited disorder with an incidence of approximately 1 in 30,000 births, caused by germ line mutations of the *NF2* gene, which is located on chromosome 22q12. The disease is characterized mainly by the development of bilateral Schwann cell tumors of the eighth cranial nerve. Mutations and loss of heterozygosity (LOH) of the *NF2* locus have been detected at high frequency in

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_2,

various tumors of the nervous system, including schwannomas, meningiomas, and ependymomas, indicative of classical tumor suppressor gene pattern (Gusella et al. 1996, 1999). In further support of a role for *NF2* in tumor suppression, mice heterozygous for an *Nf2* mutation are predisposed to a wide variety of tumors, while mice with both *Nf2* alleles inactivated specifically in Schwann cells develop schwannomas and Schwann cell hyperplasia (McClatchey et al. 1998; Giovannini et al. 1999, 2000).

The *NF2* tumor suppressor gene encodes a 69-kDa protein called Merlin (*Mo*esin, *e*zrin, and *r*adixin-*li*ke protei*n*). Merlin contains an N-terminal FERM domain that comprises three subdomains organized into a cloverleaf-like structure (Shimizu et al. 2002; Pearson et al. 2000), followed by a coiled-coil domain and a charged C-terminal tail. The *NF2* allele is alternatively spliced resulting in two predominate forms of the Merlin protein (isoform 1 and 2) that differ at the extreme C-terminus. Several studies indicated that Merlin forms intramolecular associations between the C-terminal tail and the FERM domain, transitioning between "open" and "closed" conformation (Sher et al. 2012; Gutmann et al. 1999; Sherman et al. 1997). While it has been long thought that the "closed" state represents the active/growth-suppressive form of Merlin, recent studies have cast doubt on this model (Sher et al. 2012; Hennigan et al. 2010; Lallemand et al. 2009a; Schulz et al. 2010). In particular, a study using Merlin mutants that adopt open or closed forms demonstrated that the open form is the active form of the protein (Sher et al. 2012).

A number of factors have been shown to regulate Merlin activity, including phosphorylation on serine 518 in the C-terminal tail. This phosphorylation is induced by the small G-proteins, Rac1 and Cdc42, and mediated by the immediate Rac/Cdc42 effectors—the p21-activated kinases (Paks) (Kissil et al. 2002; Xiao et al. 2002). In addition, it was shown that cAMP-dependent kinase (PKA) also phosphorylates Merlin at serine 518 (Alfthan et al. 2004). An additional level of regulation is provided by the myosin phosphatase MYPT1-PP1, which dephosphorylates Merlin at serine 518 (Jin et al. 2006). Finally, AKT was shown to phosphorylate Merlin at serine 10, threonine 230, and serine 315, promoting its proteosomal degradation (Tang et al. 2007; Laulajainen et al. 2011).

2.2 Merlin Localization and Function

Merlin is localized predominantly to membrane periphery within cells. As cells reach confluence, Merlin is recruited to cell junctions, most likely through interactions with α-catenin (adherens junctions, AJs) or Amot (tight junctions, TJs), where it is thought to coordinate the establishment of intercellular contacts with concomitant inhibition of proliferative signaling (Curto et al. 2007; Rangwala et al. 2005; Lallemand et al. 2003; Morris and McClatchey 2009; Gladden et al. 2010; Yi et al. 2011). In vitro and in vivo studies using different experimental systems have yielded conflicting results on whether or not Merlin is required for the assembly or maintenance of cell junctions (Lallemand et al. 2003; Morris and McClatchey 2009; Gladden et al. 2010; Houshmandi

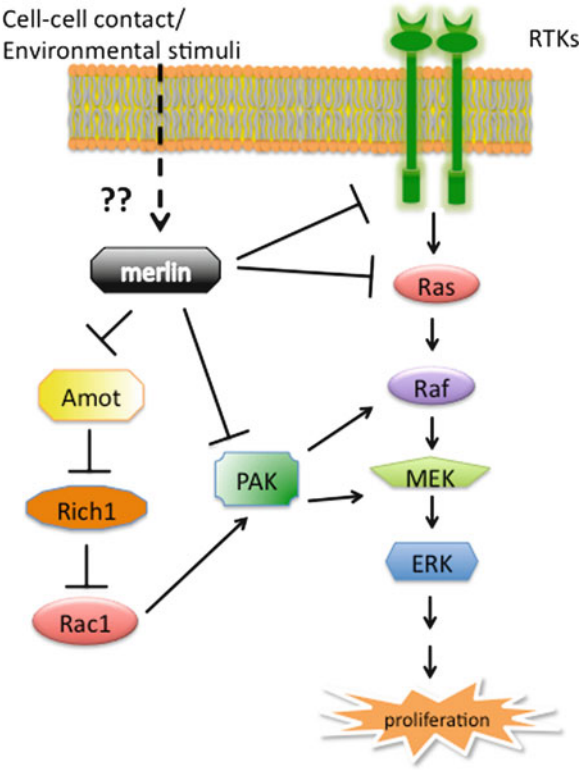

Fig. 2.1 Schematic representation showing the modes through which Merlin regulates Rac and Ras signaling

et al. 2009; Lallemand et al. 2009b; McLaughlin et al. 2007; Flaiz et al. 2009; Okada et al. 2005). Nevertheless, the localization of Merlin to cell junctions appears to be critical for its tumor-suppressive function, as patient-derived mutations that impair Merlin's junctional localization render the protein inactive (Lallemand et al. 2003; Gutmann et al. 2001; Stokowski and Cox 2000; Deguen et al. 1998).

While Merlin has also been shown to have nuclear functions (Li et al. 2010), the vast majority of evidence implicates Merlin in mediating contact-dependent inhibition of cell proliferation from the cell membrane, by coupling signals initiated through cell–cell interactions with regulation of growth regulatory pathways, including the Ras and Rac, Src, mTOR, and Hippo-Yap pathways (Huson et al. 2011). In particular, numerous studies have linked Merlin to the Ras and Rac signaling pathways (Fig. 2.1) (Yi et al. 2011; Okada et al. 2005; Kaempchen et al. 2003; Morrison et al. 2007; Wong et al. 2012; Zhou et al. 2011a; Hennigan et al. 2012; Bosco et al. 2010). The Ras and Rac protein families are small G-proteins that function as molecular switches cycling between an "ON" state when bound to GTP and an "OFF" state when bound to GDP. They are tightly regulated by various groups of proteins, including GEFs (Guanine Exchange Factors), which promote binding of

small GTPases to GTP, and GAPs (GTPase Activating Proteins) that promote the hydrolysis of GTP to GDP. The transition from an "ON" to an "OFF" state is regulated by various stimuli, including growth-factor receptors and integrins (Burridge and Wennerberg 2004). Ras is a well-documented oncogene that is mutated in a significant number of cancers. However, the roles of the Rac family of proteins in cancer have not been fully elucidated. Both Ras and Rac protein families are master regulators of diverse signaling pathways that control the shape, motility, and growth of cells. These are processes that often go awry in cancer. While activating Rac mutations have not been found in tumors, there is strong evidence that Rac plays a crucial role in the regulation of signaling cascades downstream of Ras. One of the main mechanisms demonstrated is through the phosphorylation of c-Raf (serine 338) and MEK1 (serine 298) by Paks following Rac1 activation by Ras, which is required for the sustained activation of the MAPK signaling by Ras (Fig. 2.1) (Sun et al. 2000; Howe and Juliano 2000; Diaz et al. 1997; Frost et al. 1997; King et al. 1998; Vadlamudi et al. 2000).

Merlin has been shown to regulate mitogenic signaling at multiple levels. Recent studies suggested that loss of Merlin leads to accumulation of receptor tyrosine kinases (RTKs) at the cell surface, possibly due to defects in receptor trafficking (Lallemand et al. 2009b; Ammoun et al. 2008; Maitra et al. 2006), or by sequestering them to microdomains of the plasma membrane (Curto et al. 2007; Morris and McClatchey 2009; Cole et al. 2008). Downstream of RTKs, Merlin was shown to also inhibit the activation of the small GTPases Ras and Rac1 (Okada et al. 2005; Morrison et al. 2007). Many Ras-controlled pathways are upregulated in human schwannomas (Ammoun et al. 2008) and mechanistic studies suggested that Merlin regulates Ras signaling by disrupting a Grb2-SOS-ERM-Ras complex, leading to lower levels of activated Ras (Morrison et al. 2007). Previous work by multiple groups demonstrated that Merlin functions to prevent Rac1-mediated activation of Paks by interfering with the binding of activated Rac1 to Pak1 (Kissil et al. 2003; Xiao et al. 2005; Hirokawa et al. 2004). Merlin also acts upstream of Rac1, as expression of dominant-active Rac1 as well as dominant-active Pak prevents Merlin from inhibiting Ras-induced activation of MAPK signaling (Morrison et al. 2007). Recent work added insights into the mechanisms of how Merlin functions upstream of Rac1 and Ras-MAPK signaling through inhibition of Rich1, a Rac1/Cdc42 GAP (see below) (Yi et al. 2011). In addition, Merlin was shown to inhibit contact-dependent recruitment of active Rac1 to the plasma membrane in endothelial cells (Okada et al. 2005).

Genetic studies in both flies and mice demonstrated that the Hippo-Yap pathway is a key effector pathway mediating the tumor-suppressive function of Merlin (Zhang et al. 2010; Hamaratoglu et al. 2006). In this chapter, we will discuss our current understanding of how Merlin and its major interacting partners, the Angiomotins, modulate the Hippo-Yap pathway. The reader is referred to other reviews for details relating to Merlin's regulation of other signaling pathways (Li et al. 2012a; Zhou and Hanemann 2012).

2.3 The Angiomotins: Novel Merlin Interacting Proteins

While numerous proteins have been identified as merlin-interacting proteins over the past decade, it is the Angiomotins that have been directly implicated in the tumor-suppressive function of Merlin as well as regulation of the Hippo-Yap pathway (Yi et al. 2011; Varelas et al. 2010; Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011; Paramasivam et al. 2011; Oka et al. 2012). As members of the Motin protein family, Angiomotin (Amot), Angiomotin-like 1 (AmotL1), and Angiomotin-like 2 (Amotl2) are characterized by a conserved N-terminal glutamine-rich domain, followed by a coiled-coil domain and a C-terminal PDZ-binding motif (Fig. 2.2) (Bratt et al. 2002). Angiomotin, the founding member of the Motin family has two major splice forms (p80 and p130 isoforms) and was originally isolated as an Angiostatin binding protein that mediates the anti-migratory properties of Angiostatin (Bratt et al. 2005; Troyanovsky et al. 2001). Interestingly, all members of the Motin family appear to associate with TJs through binding to the TJ-associated Patj/Mupp1 proteins (Wells et al. 2006; Sugihara-Mizuno et al. 2007; Ernkvist et al. 2009).

The Angiomotins have been extensively studied in the context of angiogenesis during development. Knocking down or deleting Motin family members individually or in combination results in defects in endothelial cell polarization, migration, and proliferation of various severities, suggesting that these proteins have overlapping functions in promoting angiogenesis (Ernkvist et al. 2009; Huang et al. 2007; Aase et al. 2007; Garnaas et al. 2008; Zheng et al. 2009; Wang et al. 2011b). The roles of Amot in mediating endothelial cell polarization and migration require its C-terminal PDZ binding motif, which mediates its indirect association with Syx, a RhoA-specific GEF, via Patj/Mupp1 (Ernkvist et al. 2009; Garnaas et al. 2008). In addition, Amot was shown to preferentially bind to mono-phosphorylated phosphatidylinositols and mediate endocytic recruitment of Patj/Mupp1 and Syx (Heller et al. 2010; Wu et al. 2011). It has been suggested that Amot might coordinate cell migration and junctional remodeling by trafficking Syx together with TJ proteins Patj/Mupp1 through endocytic vesicles to the leading edge of migrating endothelial cells, leading to focal activation of RhoA at the leading edge (Ernkvist et al. 2009; Wu et al. 2011).

In addition to its role in regulating local activity of RhoA, multiple studies demonstrated that Amot inhibits Rac1 and Cdc42 activities by bindings to and inhibiting the function of Rich1, a Rac1/Cdc42 GAP localized to TJs and AJs in epithelial cells (Yi et al. 2011; Wells et al. 2006). We previously showed that Merlin, through competitive binding to Amot, releases Rich1 from an Amot-inhibitory complex, allowing Rich1 to inactivate Rac1, ultimately leading to attenuation of Rac1 and Ras/MAPK signaling (Yi et al. 2011). Moreover, Merlin mutants that carry mutations found in NF2 patients showed diminished binding capacities to Amot and were unable to dissociate Rich1 from Amot or inhibit MAPK signaling (Yi et al. 2011). The depletion of Amot in *Nf2*$^{-/-}$ Schwann cells attenuated the Ras-MAPK signaling pathway, impeded cellular proliferation in vitro and tumorigenesis

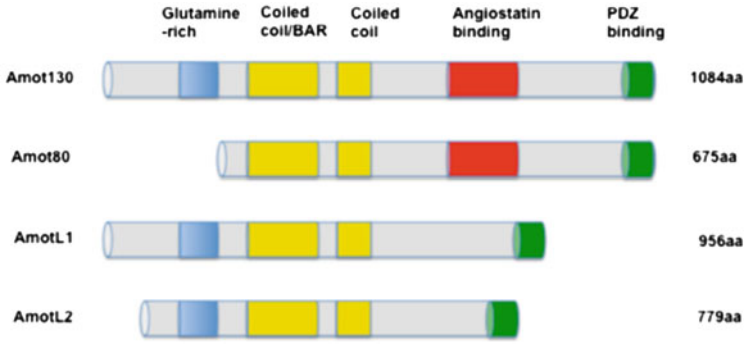

Fig. 2.2 The Motin protein family. Angiomotin p80 (Amot80) is an N-terminal truncated version of Angiomotin p130 (Amot130) as a result of alternative splicing. Angiomotin-like proteins 1 and 2 (Amotl1 and Amotl2) share sequence identity to Amot p130 but notably they lack the angiostatin-binding domain

in vivo (Yi et al. 2011). Consistent with our findings, Amot and AmotL2 were later reported to promote MAPK signaling and cell proliferation in human mammary epithelial cells, human umbilical vein endothelial cells, and zebrafish embryos (Wang et al. 2011b; Ranahan et al. 2010). Similarly, knockdown of AmotL1 was shown to decrease pERK and pAKT levels in MCF10A cells (Wang et al. 2011a). Interestingly, the same study found that silencing of AmotL2 has opposite effects on pERK and pAKT levels (Wang et al. 2011a). Further investigation will be required to reconcile these findings with previous reports.

Finally, the Angiomotins were recently identified as major binding partners for Yap (Varelas et al. 2010; Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011; Paramasivam et al. 2011; Oka et al. 2012). We will discuss in detail below the various mechanisms that have been proposed of how the Angiomotins intercept with Hippo-Yap signaling and their relationship to Merlin.

2.4 The Hippo-Yap Signaling Pathway

The Hippo-Yap pathway is described in great detail in other chapters of this book. Briefly, this pathway was initially characterized in flies and shown to play a role in organ size control. Subsequent studies indicate that the pathway is a mediator of cell contact inhibition and tumor suppression (Ota and Sasaki 2008; Zeng and Hong 2008). The pathway is composed of a core kinase cascade, in which the Mst1/2 kinases (Hippo in flies) in complex with scaffold protein WW45 (Salvador in flies) phosphorylate Lats1/2 kinases (Warts in flies) and their adaptor protein, Mob1 (Mats in flies). Phosphorylated Lats1/2 in turn phosphorylate Yap (Yorkie in flies), a transcriptional co-activator. The phosphorylation of Yap not only prevents it from

entering into the nucleus but also primes it for ubiquitination and degradation by the proteasome (Zhao et al. 2007, 2010). Upon dephosphorylation likely by PP1A (Wang et al. 2011c; Liu et al. 2011), Yap translocates into the nucleus where it complexes with Tead (Scalloped in flies) and other transcription factors to drive the expression of pro-proliferative or anti-apoptotic genes (Hong and Guan 2012). A series of recent studies have demonstrated that, akin to what has been observed in flies, the mammalian Hippo pathway also regulates organ size, particularly of the liver. Inducible overexpression of Yap in adult mouse liver results in rapid and reversible increase in liver size (Camargo et al. 2007; Dong et al. 2007). Comparable hepatomegaly phenotypes were observed when *Mst1/2, WW45* and *Nf2* were ablated specifically in the liver (Zhang et al. 2010; Zhou et al. 2009, 2011b; Benhamouche et al. 2010; Song et al. 2010; Lee et al. 2010; Lu et al. 2010). Finally, increased Yap activity appears to be a common occurrence in human hepatocellular carcinoma (Zhou et al. 2009; Li et al. 2012b; Xu et al. 2009; Zender et al. 2006).

2.5 Merlin and Hippo-Yap Signaling

Merlin and another FERM domain protein, Expanded, were first identified as upstream regulators of Hippo signaling through genetic screens in Drosophila (Hamaratoglu et al. 2006). Merlin regulates the expression and localization of Yap in mammalian cells in a manner similar to what has been observed in flies (Zhao et al. 2007; Striedinger et al. 2008; Yokoyama et al. 2008). It was reported that majority of human meningioma and mesothelioma samples, which are frequently associated with loss of *NF2*, exhibited elevated Yap expression in the nuclei, indicative of abnormal Yap activation (Zhao et al. 2007; Striedinger et al. 2008; Baia et al. 2012; Sekido 2011). Knockdown of Yap was shown to rescue the hyperproliferative phenotype of *NF2*-deficient meningioma and mesothelioma cells, whereas hepatomegaly/tumorigenesis phenotypes associated liver-specific *Nf2* deletion in mice were largely suppressed by concomitant heterozygous deletion of *Yap* or overexpression of a dominant-negative Tead (Zhang et al. 2010; Striedinger et al. 2008; Baia et al. 2012; Mizuno et al. 2012; Liu-Chittenden et al. 2012). These studies thus validated the Yap/Tead transcriptional complexes as major downstream effectors of Merlin/*NF2* in growth regulation in mammals.

The exact mechanisms of how Merlin regulates Hippo signaling and Yap activity remain to be elucidated. In Drosophila, Merlin was shown to form an apical complex with Kibra and together they activate Sav and Wts, leading to Yki phosphorylation and inactivation (Yu et al. 2010; Genevet et al. 2010; Baumgartner et al. 2010). Yeast two hybrid and biochemical studies indicated that Merlin directly binds to Sav through its N-terminal FERM domain and Kibra through its C-terminal half (Yu et al. 2010). Interestingly, the physical and functional interactions between Merlin, Kibra, and WW45 (the mammalian counterpart of Sav) appear to be conserved in mammalian cells (Yu et al. 2010).

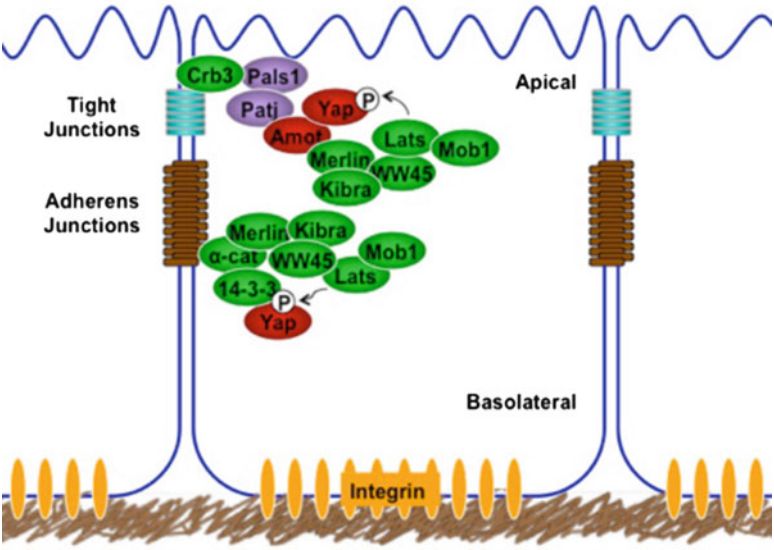

Fig. 2.3 Schematic representation of putative signaling complexes assembled by Merlin at the cell junctions. In *green* are proteins previously implicated as having growth/tumor-suppressive functions, in *red* proteins implicated as having pro-proliferative functions and in *purple* proteins with yet to be defined functions

In addition to Hippo signaling components, Merlin has been reported to function as a linker between the AJ-associated catenin complex and the TJ-associated Par3 complex by directly binding to α-catenin and Par3 (Gladden et al. 2010). Interestingly, two recent studies demonstrated that Yap also associates with α-catenin through 14-3-3 in a phosphorylation-dependent manner (Silvis et al. 2011; Schlegelmilch et al. 2011). Besides the catenin complex, both Merlin and Yap interact with TJ-associated Crumbs-Pals1-Patj polarity complex via direct binding to distinct domains of the Angiomotins (Yi et al. 2011; Varelas et al. 2010). Taken together, it is tempting to speculate that Merlin together with the junctional complexes may promote Hippo signaling and Yap inactivation by assembling signaling platforms where Lats1/2 kinases, in response to signals from the junctional complexes, phosphorylate and inactivate Yap (Fig. 2.3).

2.6 Angiomotin and Hippo-Yap Signaling

Recent work has also implicated the Angiomotin family members in the regulation of Hippo-Yap signaling (Varelas et al. 2010; Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011; Paramasivam et al. 2011; Oka et al. 2012). At least under conditions used for immunoprecipitation, the Angiomotins are arguably one of the

strongest binding partners for Yap, as evidenced by four independent studies identifying them as major Yap-associated proteins (Varelas et al. 2010; Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011). The interactions between the Angiomotins and Yap are mediated through PPXY motifs within the N-terminal regions of the Angiomotins and the conserved WW domains of Yap (Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011). Interestingly, several other proteins including Lats1/2 also bind to the WW domains of Yap through their PPXY motifs (Chen and Sudol 1995; Strano et al. 2001; Komuro et al. 2003; Hao et al. 2008; Espanel and Sudol 2001). Therefore, it would be interesting to test whether Angiomotins may regulate their interactions with Yap.

Largely based on overexpression of the Angiomotins in HeLa and MCF7 cells, which do not appear to express these proteins endogenously, several recent studies suggested that the Angiomotins function as negative regulators of Yap and its paralog Taz by sequestering them in the cytoplasm (Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011). It should be noted, however, that exogenously expressed Angiomotins in these cells form mainly cytoplasmic aggregates, instead of localizing to the cell junctions (Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011). This raises the question of whether these approaches accurately reflect the physiological functions of the Angiomotins. Significantly, stable overexpression of Amot-p130 in MDCK cells, where it is correctly targeted to TJs, leads to increased localization of endogenous Yap not only to the junctions but also within the nucleus (Zhao et al. 2011). Loss of function studies by knocking down members of the Motin family in cells that express endogenously at least one of three members of the family demonstrated that silencing of AmotL2 but not AmotL1 increased the localization of Yap and Taz to the nucleus and induced cellular transformation in MDCK and MCF10A cells (Zhao et al. 2011; Wang et al. 2011a; Chan et al. 2011). In addition, knockdown of AmotL2 or AmotL1 appeared to have opposite effects on MAPK signaling in MCF10A cells (Wang et al. 2011a). Notably, the observed effect of AmotL2 silencing on MAPK signaling in MCF10A cells was in contrast to another knockdown study in zebrafish embryos and HUVEC cells (Wang et al. 2011a, 2011b). It is possible that these discrepancies are due to tissue- or cell-type-specific functions of different members of the Motin family. Further studies will be necessary to clarify these differences.

In addition to controlling Yap subcellular localization, the Angiomotins have been reported to promote Yap phosphorylation and diminish transcription of two known Yap target genes, CTGF and Cyr61 (Zhao et al. 2011; Wang et al. 2011a; Paramasivam et al. 2011). One study suggested that the Angiomotins bind to and enhance the kinase activity of Lats1/2, thereby increasing Yap phosphorylation (Paramasivam et al. 2011). One potential caveat is that this study was performed primarily with overexpressed Angiomotins tagged at their C-terminus, which may mask their C-terminal PDZ binding domains (Fig. 2.2) and hinder their binding to partners such as Patj/Mupp1, thus potentially interfering with the normal functions of the Angiomotins. Given that other studies have shown that the Angiomotins can regulate Yap activity independent of Hippo signaling, the precise functions of the Motin family members in Hippo signaling remain to be defined.

Conflicting data also exist regarding to the roles of the Motin family in tumorigenesis. We previously showed that Amot functions downstream of Merlin as a positive regulator of Rac1 and MAPK signaling in vitro and is required for tumorigenesis following Merlin/*Nf2* loss in vivo, using an orthotopic model of NF2 (Yi et al. 2011). This finding of a pro-proliferative role for Amot was corroborated by two other studies in mammary epithelial cells and zebrafish embryos (Wang et al. 2011b; Heller et al. 2010). Other reports suggested that knockdown of AmotL2 leads to cellular transformation in MDCK and MCF10A cells in vitro (Zhao et al. 2011; Wang et al. 2011a). It is possible that this is a reflection of different roles carried out by members of the Motin family and/or a choice of model system used in the studies. The availability of knockout and transgenic mouse models in which components of the Hippo-Yap pathway have been targeted will no doubt facilitate the definition of the roles the Motin family plays in tumorigenesis (Zhang et al. 2010; Camargo et al. 2007; Dong et al. 2007; Zhou et al. 2009, 2011b; Benhamouche et al. 2010; Song et al. 2010; Lee et al. 2010; Lu et al. 2010; Schlegelmilch et al. 2011). For example, the role of Amot in tumorigenesis could be addressed, in vivo, by combining the Amot conditional knockout allele (Shimono and Behringer 2003) with knockout or transgenic alleles of Hippo-Yap pathway components in a tractable system such as the liver and examining the effects of Amot knockout on hepatomegaly and tumorigenesis phenotypes caused by perturbance of Merlin or the Hippo-Yap pathway. These studies in a physiological system should help define the relationship between Merlin, the Motin family, and the Hippo-Yap pathway.

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Chapter 3
MST1/2 and Other Upstream Signaling that Affect Hippo Pathway Function

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Abstract The Hippo signaling pathway is a conserved regulator of proliferation and cell survival in metazoans. The core of the pathway consists of a kinase cascade with the tumor suppressor Hippo (and its mammalian orthologues, MST1 and MST2) as the upstream kinase. Hippo activation is coupled to diverse stimuli, including cell contact, cell stress, and growth factor receptor signaling. These stimuli engage the Hippo pathway via multiple different signaling mechanisms that are cell type and cell context dependent. Notably, distinct mechanisms that control Hippo signaling have been uncovered in hematopoietic, epithelial, and mesenchymal lineages, and in cells exposed to strong inducers of apoptosis. Here we provide an overview of the signaling processes controlling mammalian Hippo pathway function, focusing on the direct regulation of MST1 and MST2 activity.

Keywords Hippo pathway • Mst1/STK4 • Mst2/STK3 • WW45 • SARAH domain • Mob1 • Lats1/2 • YAP • T cells • Liver • Cancer

3.1 Hippo Signaling in Mammals

The Hippo signaling pathway was first identified in *Drosophila* through genetic screens for genes controlling of tissue growth. At the core of this signaling cascade is the Hippo kinase, which functions as a tumor suppressor and regulator of organ size. Hippo binds and phosphorylates the non-catalytic scaffold protein Salvador (Sav, also known as Shar-pei), facilitating Hippo-mediated phosphorylation and activation of the Warts (Wts) kinase. The latter acts with the adaptor protein Mats by direct binding. This cascade culminates in the Wts-mediated phosphorylation of

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_3,

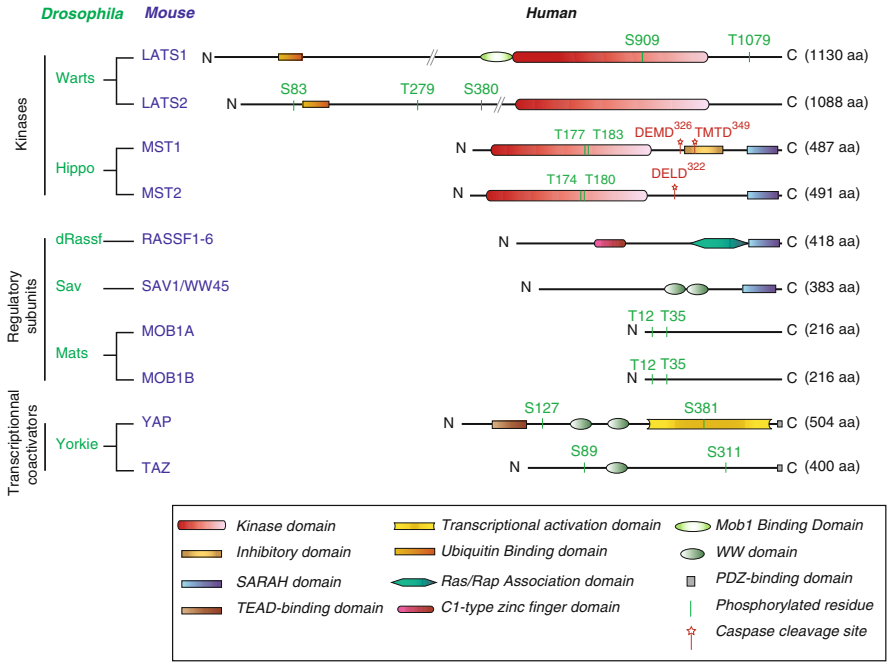

Fig. 3.1 Structure of core components of the mammalian Hippo pathway

the oncogenic transcriptional coactivator Yorkie (Yki). As a result, Yki is bound by 14-3-3 proteins leading its cytoplasmic retention and consequent inactivation. Yki is the critical downstream target of Hippo signaling as the overgrowth phenotypes observed in the Hippo, Sav, or Wts loss-of-function mutants are associated with the constitutive nuclear localization of Yki, and correspondingly Yki inactivation rescues these phenotypes. Yki's function requires association with TEA-domain (TEAD) family DNA-binding transcription factors, including Scalloped (Sd), Homothorax (Hth), and Teashirt (Tsh). Key target genes activated by Yki include *Cyclin E*, *DIAP1*, and the microRNA *bantam*, which confer increased proliferation and defects in developmental apoptosis.

All of the core components of the Hippo signaling pathway are conserved and duplicated in mammalian genomes, except for Sav and Yki that are present as single orthologues in vertebrates, (SAV1/WW45 and YAP, respectively), although YAP has a paralogue, TAZ, which is also negatively regulated by this pathway (Fig. 3.1). These components in mammals form a kinase cascade similar to that observed in flies, and several loss-of-function mutant phenotypes observed in *Drosophila* can be rescued by the expression of their human counterparts. Indeed, the sterile 20-like kinases MST1/STK4 and MST2/STK3, orthologues of the *Drosophila* Hippo kinase, interact with their regulatory subunit SAV1 to form an active complex that can phosphorylate and activate the kinases LATS1 and LATS2, the direct orthologues

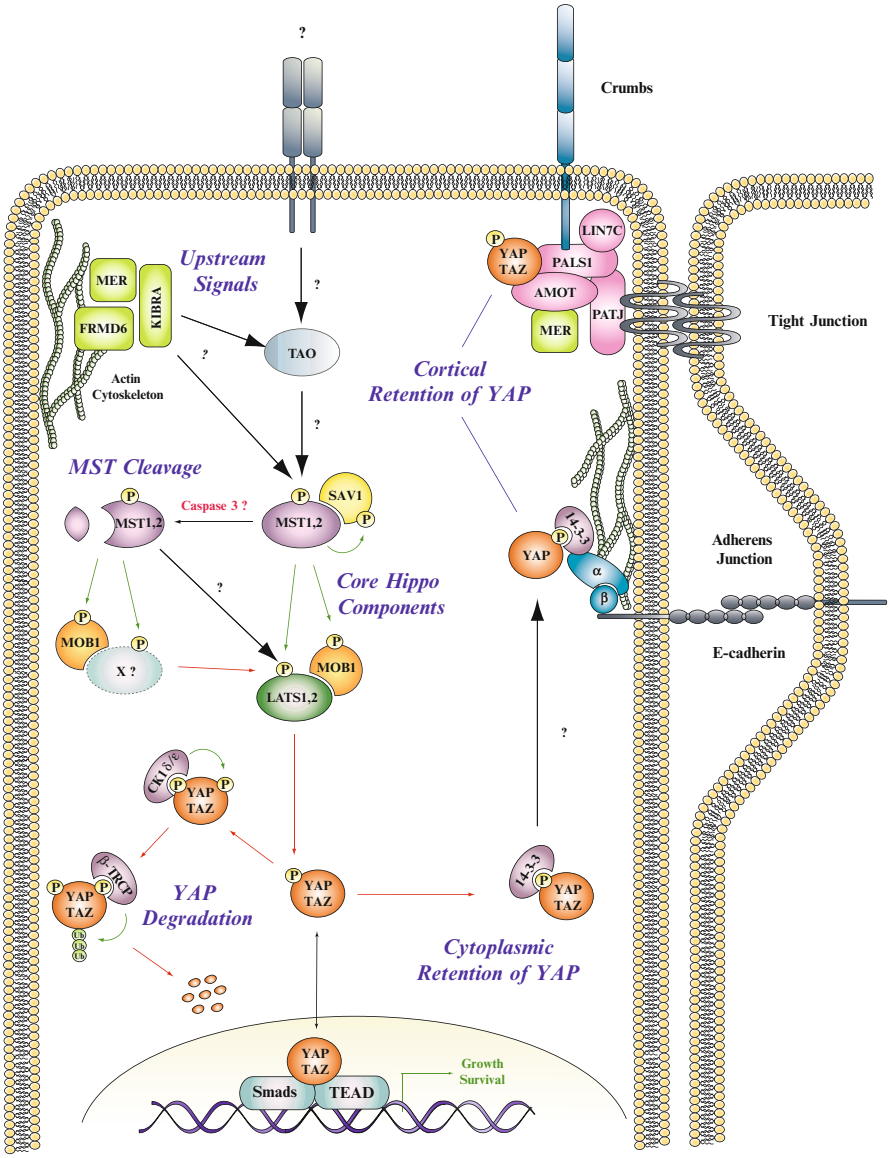

Fig. 3.2 Model of Hippo pathway circuitry in mammalian epithelial cells

of Warts (see Core Hippo components in Fig. 3.2). LATS1,2 are regulated by the Mats orthologues, MOBKL1A/B (collectively referred to as MOB1), which are also phosphorylated by MST1/MST2 to enhance binding in the LATS1,2-MOB1 complex (Praskova et al. 2008). Activated LATS1,2 phosphorylates the transcriptional coactivator YAP on five different consensus HXRXXS motifs (see YAP degradation

and YAP cytoplasmic retention in Fig. 3.2). As for Yki in Drosophila, the phosphorylation of YAP at Ser127 (Ser89 for TAZ) promotes their cytoplasmic retention through 14-3-3 binding. Moreover, phosphorylation of Ser381 primes YAP for subsequent phosphorylation by CK1δ/ε in a phosphodegron, thereby promoting the recruitment of the SCF $^{\beta\text{-TRCP}}$ E3 ubiquitin ligase which catalyzes YAP ubiquitination, ultimately leading to proteosomal degradation of YAP (Zhao et al. 2010). Unphosphorylated YAP/TAZ primarily localize in the nucleus, where they interact with DNA-binding transcription factors such as TEAD1-4 or SMAD proteins, in order to regulate a transcriptional program that promotes cell survival and proliferation. It is important to note that in addition to the strong conservation of Hippo pathway components and signaling relationships, it is also clear that mammalian Hippo signaling has additional complexity with distinct inputs and downstream targets in specific tissues and contexts. Thus, depending on the setting, MST1/2 can be activated independently of SAV1 and can regulate growth through downstream targets other than LATS1/2-YAP; likewise, inputs other than MST1/MST2 may regulate LATS1/2.

3.2 The "Hippo" Kinases, Hippo, MST1, and MST2

MST1 was identified using degenerate PCR for Ste20-related kinases (Creasy and Chernoff 1995a, b) and, together with MST2, as a kinase activated by cell stress (e.g., arsenite, heat shock) or okadaic acid, as detected by "in-gel" kinase assays of cell extracts (Taylor et al. 1996; Wang and Erikson 1992). The MST1/MST2 catalytic domain shows homology with the Germinal Center kinase (GCK) subgroup of the Ste20 kinase family. The GCKs can be divided into eight subfamilies, all containing an Ste20-related catalytic domain beginning near their N-terminus, and distinguished by unique C-terminal sequence and domain structures (Dan et al. 2001). Drosophila Hippo (669 amino acids), MST1 (487AA) and MST2 (491AA) (~75 % identical to each other) are members of group II GCKs, with a domain structure distinguished by a specialized coiled coil domain near the N-terminus (Hippo AA607-655) known as a SARAH domain, an acronym designating the three gene families that contain homologous domains, i.e., Salvador/SAV1, RASSF(1–6) and Hippo/MST1/MST2 (Scheel and Hofmann 2003). The SARAH domain of MST1 (AA432-480) forms a hairpin of helical segments, with the short N-terminal helical segment (AA 433–437) bent back ~45° toward the C-terminal helix (AA441–480); the latter mediates the formation of SARAH/SARAH homo/heterodimers through an antiparallel, head-to-tail interaction stabilized by hydrophobic residues (Avruch et al. 2009). As discussed in further detail below, heterodimerization of MST1 and MST2 with the non-catalytic RASSF1-6 or SAV1 polypeptides via their respective SARAH domains couples MST1/MST2 activation to distinct upstream inputs in different tissues (Fig. 3.1 shows the domain structure of each of these proteins).

3.3 Hippo/MST1/MST2 Regulation

3.3.1 Autoactivation

When MST1 or MST2 are transiently overexpressed and immunoprecipitated, they are found primarily as a dimer of the full length polypeptides. MST1/MST2 catalytic activity is markedly increased in vitro by the presence of Mg+ATP that enhances auto-transphosphorylation of the MST1 (at Ser183) or MST2 (at Ser180) activation loop within the homodimer. Co-expression of ATP-binding site mutants (MST1[K59R] or MST2[K56R]) with the wild type isoforms results in dimers showing phosphorylation of the activation loop of the mutant polypeptide, which are nevertheless inactive due to failure of transphosphorylation of the WT activation loop (Praskova et al. 2004). MST1[K59R] (or MST2[K56R]) thus act as dominant inhibitors (Praskova et al. 2008). Impairment of homodimerization via deletion of the SARAH domain or site-specific mutations (e.g., MST1[L444P]) strongly attenuate autoactivation in vitro (Praskova et al. 2004). MST1 has an autoinhibitory domain as reflected by the markedly increased kinase activity of transiently expressed isoforms with deletions between the MST1 catalytic domain and the SARAH domain, e.g., Δ331–394 (Creasy et al. 1996).

3.3.2 Upstream Regulation of the Hippo Pathway

The Hippo pathway is an important sensor for relaying extrinsic stimuli with changes in cell proliferation and motility. Whereas the many regulatory inputs for the pathway and how they are integrated are incompletely understood, there appear to be common themes emerging involving distinct machinery in different tissues. Broadly, the machinery differs in hematopoietic cells from epithelial cells, and specific epithelial lineages possess their own characteristic organization of the pathway.

3.3.2.1 Regulation by RASSF Polypeptides

In hematologic cells, genetic and biochemical studies have pointed to particularly important roles for the RASSF1-6 polypeptides as immediate upstream inducers of MST1/MST2 kinase function. These non-catalytic polypeptides are members of a larger family of proteins (RASSF1-10), all containing an RAS-RAP association (RA) domain, the basis of their shared name, whereas only RASSF1-6 contain a SARAH domain and thus the ability to bind MST1/MST2 (Avruch et al. 2009; Underhill-Day et al. 2011). The founding member, RASSF5 (originally named Nore1 (Vavvas et al. 1998)), was discovered through its ability to bind preferentially and with high affinity to RAS-GTP over RAS-GDP, and subsequently to the

GTP liganded form of several other RAS-like small GTPases (Ortiz-Vega et al. 2002). RASSF(1–6) share homologous ~225AA C-terminal segments that contain an RA domain followed immediately by a SARAH domain (Fig. 3.1). RASSF1 and RASSF5 are expressed as two predominant splice variants that differ only in their N-terminal segments upstream of the RA domain. The N-termini of the longer isoforms (RASSF1A and RASSF5A/Nore1A) contain one or more proline-rich motifs upstream of a C1-type zinc finger.

The relationship of MST1/MST2 with the RASSF proteins was first suggested by unbiased two hybrid screens in which MST1 was retrieved using RASSF1A as bait. It is now apparent that RASSF1-6 all can heterodimerize with MST1/MST2 through their SARAH domain (reviewed in Avruch et al. 2009). Importantly, whereas transiently expressed MST1 or MST2 is each retrieved as a SARAH domain-mediated homodimer (Praskova et al. 2004; Creasy et al. 1996), in vitro addition of equimolar amounts of recombinant RASSF5 SARAH domain results in complete conversion to an RASSF5-SARAH/MST1-SARAH heterodimer (Hwang et al. 2007). The Kd of the MST1/RASSF5 heterodimer is in the nM range, suggesting that this is a constitutive dimer; consistent with this view, immunoprecipitates of MST1 from mouse lymphoid cells contain RASSF5B (Katagiri et al. 2006). Moreover, deletion of both MST1 and MST2 from the lymphoid compartment results in the complete disappearance of the RASSF5B polypeptide without alteration in RASSF5B mRNA abundance, indicating that complex formation is required to stabilize endogenous RASSF5B (Zhou et al. 2008; Mou et al. 2012).

The physiologic importance of interaction between MST1/MST2 and RASSF5B (the predominant RASSF5/Nore1 isoform in lymphoid tissues; also called RAPL) has been demonstrated in T cells (Fig. 3.3). Here, the constitutive complex of RASSF5 with MST1/MST2 appears to be inactive at the basal state. When the T-cell receptor is stimulated in naïve T cells, the resulting conversion of RAP1 to its GTP-bound form complexes with the RA domain of RASSF5B resulting in recruitment of RAP1-GTP-RASSF5B-MST1 from the Golgi to the immune synapse, concomitant with MST1 activation (Katagiri et al. 2003, 2004). Studies in mouse T cells lacking expression of MST1 and RASSF5B strongly support the requirement for this complex in the RAP1-GTP regulation of activation of the integrin LFA-1, referred to as "inside-out" activation (Kinashi 2007). Correspondingly, both humans and mice with inactivating mutations in MST1 exhibit multiple defects in lymphocyte adhesion and migration, and in immune function (Zhou et al. 2008; Mou et al. 2012; Katagiri et al. 2009; Dong et al. 2009; Abdollahpour et al. 2012; Nehme et al. 2012). Mice deficient in RASSF5 (RAPL) show an overlapping set of lymphoid defects (Katagiri et al. 2004, 2006).

The precise mechanism of MST1 activation in complex with RASSF5 and RAP1-GTP is not known. High levels of overexpression of any of the RASSF5 or RASSF1 polypeptides inhibits MST1[Ser183] phosphorylation in vivo and blocks the ability of MST1 to autoactivate in vitro by displacing the MST1 homodimer into an RASSF/MST1 heterodimer (Praskova et al. 2004). This behavior implies that RASSF5B/MST1 complexes are inactive in the absence of an upstream input. While co-expression of MST1 with excess of RASSF5 and activated forms of RAS does

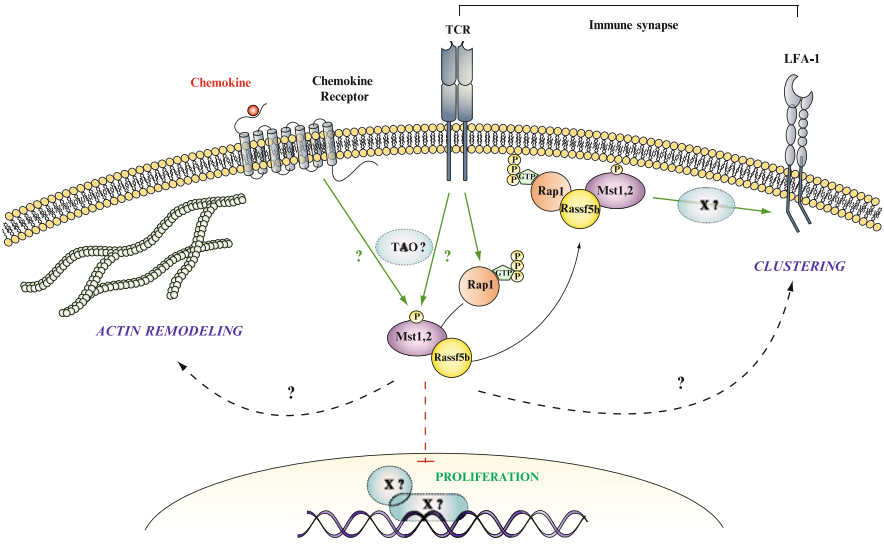

Fig. 3.3 Model of Hippo pathway circuitry in lymphocytes

not result in the activation of the bulk of MST1, the MST1 polypeptides that are in association with RAS-GTP show a increased Ser183 phosphorylation as compared to the bulk MST1 (Praskova et al. 2004). Thus, continued association of the RASSF5/MST1 complex with the activated GTPase appears to be required to maintain kinase activation. This could serve as a mechanism to constrain MST1 action and may reflect rapid dephosphorylation once freed from the GTPase. The activation of MST1 by RAS/RAP1 could be due to the membrane localization resulting in local increases MST1 concentration that facilitates transphosphorylation. Consistent with this possibility, the direct membrane targeting of MST1 via fusion with the SRC myristoylation motif is sufficient to potently activate MST1 (Praskova et al. 2004).

The relationship between the other members of the RASSF1-6 group and the regulation of MST1/MST2 is poorly understood. Early studies demonstrated that val12-RAS can associate with RASSF1 when both are overexpressed (Vos et al. 2000) and that apoptosis induced by overexpression of val12-Ki-RAS is blocked by co-expression with the SARAH domain of either MST1 or RASSF5. However, the affinity of RAS/RAP1-GTP for the RASSF1 RA domain is more than 30-fold lower than for RASSF5B RA domain (Wohlgemuth et al. 2005). Correspondingly, there is presently no evidence that endogenous RASSF1A is recruited by RAS-like GTPases. In contrast to the studies mentioned above that show that MST1 is inhibited when RASSF1A and RASSF1C are co-expressed in excess of MST1 (conditions wherein all MST1 polypeptides are bound by an RASSF polypeptide) (Praskova et al. 2004), several groups have reported that transient expression of RASSF1A, but not the shorter RASSF1C, can result in activation of endogenous or co-expressed MST1/MST2 (Guo et al. 2007; Oh et al. 2006; Matallanas et al. 2007). Whether these

differing results reflect relative expression levels or other experimental conditions is not known. With regard to the selective ability of RASSF1A to activate MST1, this activity must depend on its unique N-terminal segment as RASSF1A and RASSF1C share identical RA and SARAH domains and bind MST1 comparably. Assuming that, as for the RASSF5B/MST1 complex, upstream inputs exist that can activate RASSF1A-bound MST1/2, activation of MST1 by RASSF1A may occur through the binding of the RASSF1A N-terminal segment to an element that activates the bound RASSF1A/MST1-2 complex. Alternatively, the activation of MST1 by RASSF1A might occur indirectly, e.g., through an ability of RASSF1A to trigger more broad cellular processes that activate MST1/MST2, such as those that are recruited during apoptosis. In this regard, RASSF1A has been implicated in the mechanism by which FAS and TNFα promote both the activation of MST1/MST2 and apoptosis (Oh et al. 2006; Matallanas et al. 2007; Vichalkovski et al. 2008). Depletion of RASSF1A by RNAi impairs FAS- or TNF-R1-induced activation of MST1/MST2 activation and also partially inhibits the apoptotic response; similarly, depletion of MST1/MST2 also reduces the apoptotic response. The ligand-mediated activation of either the TNF1-R1 or DR4 death receptors can result in recruitment of RASSF1A and in increased MST1/MST2 kinase activity. However, this recruitment is likely to involve indirect mechanisms as it is only observed several hours after receptor activation (Vichalkovski et al. 2008; Foley et al. 2008). Since apoptosis recruits many amplifiers in a feed-forward, self-magnifying cascade, the contribution of apoptosis itself to MST1/MST2 activation by death receptors is unclear. The very delayed response of MST1/MST2 activity to death receptor stimulation, the ability of recombinant RASSF1A itself to promote apoptosis, and the ability of apoptosis, through undefined mediators, to activate MST1/MST2 leave open the mechanism of MST1/MST2 regulation downstream of death receptors and the role of RASSF1A.

3.3.2.2 Regulation by SAV1

The other SARAH domain-containing protein that directly couples MST1/MST2 to upstream signaling is SAV1 (also known as WW45), which appears to be particularly significant in epithelial lineages. Drosophila Sav (608AA) contains tandem WW domains that mediate LATS binding, and a C-terminal SARAH domain that binds to Hippo. Mammalian SAV1 (383AA; see Fig. 3.1) has a similar domain structure and binds both endogenous MST2 and LATS1 in HeLa cell extracts (Guo et al. 2007), although there is considerable divergence of the more N-terminal sequences. Co-expression of Hippo with SAV1 or LATS results in the phosphorylation of both, whereas expression of all three further enhances LATS (but not SAV1) phosphorylation, an effect requiring the Hippo and SAV1 SARAH domains (Wu et al. 2003). Similar results are seen upon co-expression of SAV1, LATS1, and MST2 (Guo et al. 2007; Park and Lee 2011). It is worth noting that the interaction of the SAV1 and MST1 SARAH domains appears to differ from that of the RASSF5/MST1 heterodimer inasmuch as the recombinant SAV1 SARAH domain perturbs a

different set of residues on MST1 than does RASSF5 (Hwang et al. 2007). Moreover, addition of the SAV1 SARAH domain does not alter the binding of the RASSF5/MST1 SARAH heterodimer as detected by NMR, nor is a heterotrimer detectable upon cross-linking or gel filtration (Hwang et al. 2007). Hence, the affinity of the RASSF5 SARAH domain for MST1 is likely to be substantially higher than that of the SAV1 SARAH domain. In addition, these findings predict that complexes of MST1 with RASSF5 and with SAV1 will be mutually exclusive when SAV1 is present in excess of RASSF5. Consistent with this, immunoprecipitates of either Salvador or dRassf from extracts of *Drosophila* Kc cells each contain Hippo, but not each other, nor is a trimeric complex detectable when all three recombinant polypeptides are co-overexpressed (Polesello et al. 2006). However co-immunoprecipitation of endogenous SAV1 and RASSF1A from extracts of HeLa (Guo et al. 2007) and H1792 lung cancer cells (Donninger et al. 2011) has been reported. Thus, RASSF1A may interact differently with SAV1/MST1-2 than does RASSF5 (although direct comparisons have not been reported) and further studies will be needed to unravel the interactions between other RASSFs and SAV1 and MST1-2, to determine whether MST1 or MST2 heterotrimers involving SAV1 and RASSF2,3,4, or 6 do exist (e.g., (Cooper et al. 2009)) and if so, to define their regulation and output. Evidence from mouse studies so far suggests that RASSF5/MST1 and SAV1/MST1 complexes occur in different tissues and respond to entirely distinct upstream inputs.

The role of SAV1 in the regulation of MST1 and LATS1/2 has been examined in mouse primary embryonic keratinocytes, which can be induced to differentiate by addition of Ca^{++} at mM levels. Ca++ induction results in increased MST1/2[Ser183/180] phosphorylation, formation of an MST1-LATS1/LATS2-SAV1 complex, and LATS1/LATS2 and YAP phosphorylation. In addition, MST1 exhibits increased nuclear localization, associating with LATS1/2 which is constitutively in the nucleus, and YAP relocalizes to the cytoplasm presumably due to its phosphorylation and binding of 14-3-3. Embryonic keratinocytes from SAV1 knockout mice fail to show these responses, whereas normal behavior can be restored by expression of wild type SAV1 in a manner requiring an intact SARAH domain (Lee et al. 2008). Hence, at least in keratinocytes, the formation of the SAV1/MST1-2/LATS1-2 complex is itself a regulated rather than a constitutive process, and moreover, SAV1 is required for the physiologic activation of MST1/MST2 (see Core Hippo Components in Fig. 3.2). An important caveat regarding these conclusions, however, arises from the lack of evidence for YAP deregulation in MST1/MST2 null keratinocytes in vitro and the absence of abnormalities in mice with skin-specific deletion of MST1/MST2 in contrast to the overproliferation and formation of skin tumors caused by transgenic overexpression of YAP (Schlegelmilch et al. 2011). Additionally, the pathway does not appear to conform to this canonical model in liver. In particular, while deletion of SAV1 or of both MST1/MST2 in the liver leads to the liver tumorigenesis associated with YAP stabilization, there are a number of differences in these models (Lu et al. 2010; Zhou et al. 2009; Song et al. 2010; Avruch et al. 2011). Liver-specific MST1/MST2 knockout results in the overgrowth of both hepatocytes and of oval cells (putative progenitor cells associated with the

bile ducts) and in loss of inactivating phosphorylation of YAP (Ser127). By contrast, SAV1 deficiency causes only oval cell expansion and YAP-ser-127 phosphorylation and MST1/MST2 kinase activity are not impaired (Lee et al. 2010). These data raise questions regarding the exact coupling of SAV1 to MST1/MST2 activity in different cellular contexts.

3.3.2.3 Activation During Apoptosis and in Normal Liver

The presence of caspase 3 cleavage sites in MST1/MST2 in the segment between the kinase and SARAH domains (Fig. 3.1) enables additional levels of regulation of kinase activity (Graves et al. 1998, 2001; Lee et al. 2001; Reszka et al. 1999; Cheung et al. 2003). The sites are located just distal to the catalytic domain (MST1-DEMD^{326}; MST2-DELD^{321}; human MST1; caspase 6/7 TMTD^{349} (Song and Lee 2008)); Drosophila Hippo lacks these sites. In response to numerous proapoptotic stimuli, caspase 3-mediated cleavage induces the generation of ~36 kDa MST1/MST2 polypeptides (see MST cleavage in Fig. 3.2). These truncated forms are catalytically active, exhibiting marked increases in activation loop phosphorylation compared to full length MST1/MST2. Moreover, although the spontaneous fractional activation of full length recombinant MST1 (~4 %) or MST2 (~20 %) during transient expression is modest (Praskova et al. 2004), transiently expressed MST1 (or MST2) itself is a potent initiator of apoptosis, yielding abundant MST1 (or MST2) active catalytic fragments (Graves et al. 1998; Lin et al. 2002). Caspase cleavage alone is likely not sufficient for activation since transiently expressed truncated MST1 [1–330] has low activity and Ser183 phosphorylation. This suggests that MST1/MST2 activation precedes and likely promotes susceptibility to caspase cleavage (Praskova et al. 2004; Glantschnig et al. 2002). Overexpressed MST1/MST2 initiate apoptosis through p53- and Jnk-dependent pathways (Graves et al. 1998; Lin et al. 2002), and the activated caspase 3 may simply cleave preferentially the already activated MST1/MST2 polypeptides. The mechanism of activation of endogenous MST1/MST2 by proapoptotic stimuli activate is not known.

The caspase-cleaved, activated MST1/2 catalytic fragments show multiple differences from the full length polypeptides. The full length forms are almost entirely cytoplasmic, although they cycle through the nucleus, as shown by their nuclear retention following the treatment of cells with the Crm1 inhibitor Leptomycin, or by mutation of the dual nuclear export signals (MST1=AA361-370; AA444-451) (Lin et al. 2002; Ura et al. 2001). By contrast, the caspase cleaved 36 kDa MST1/MST2 polypeptides exhibit unimpeded nuclear access. Moreover, the caspase-cleaved MST1 fragment shows both higher catalytic activity than full length due to the loss of the autoinhibitory domain, as well as altered substrate selectivity (e.g., the cleaved form has reduced affinity for FOXO and enhanced affinity for Histone H2B (Anand et al. 2008).

The caspase cleavage of MST1/MST2 may be relevant to homeostasis of certain tissues in vivo. Notably, while MST1 and MST2 are detected exclusively as 55–60 kDa polypeptides in extracts of normal mouse spleen (or MEFs), the majority

of MST1 and a significant fraction of the MST2 polypeptides in liver extracts are observed as 36 kDa forms (Zhou et al. 2009). Immunoblot with phosphospecific antibodies directed against the MST1/MST2 activation loop ([Ser183-P]/[Ser180-P], respectively) gives weak signal in spleen and MEF extracts, whereas the liver extracts exhibit an abundant 36 kDa polypeptide, shown by immunoprecipitation to be primarily MST1. Thus normal liver contains a substantial amount of the constitutively active, monomeric (~40 kDa by gel filtration) MST1 (and some MST2) catalytic fragment. A plausible hypothesis is that these catalytically active MST1/MST2 fragments are generated by caspase 3 cleavage of a preactivated MST1; nevertheless, other markers of apoptosis and caspase 3 activation, including the 17 kDa-activated caspase 3 are not evident (but are readily elicited by FAS activation in vivo). While the mechanism by which MST1/MST2 are activated and cleaved in the liver has not been defined or directly linked to caspase 3, it is notable that nonapoptotic functions of caspase 3 are well documented, e.g., in the differentiation of several cellular lineages (Fernando et al. 2002; Murray et al. 2008; Yi and Yuan 2009). Truncated activated MST1/MST2 fragments are also detectable in extracts of mouse intestinal epithelium. The cleavage and high levels of constitutive MST1/MST2 in liver and intestine may be important for the potent anti-proliferative role for these kinases in these organs.

3.3.2.4 Regulation by AKT

Although genetic interactions between dAkt and Hippo have not been described, a number of studies suggest that AKT can act as a negative regulator of MST1 and perhaps MST2. Initial studies reported that EGF treatment of COS cells reduced the kinase activity of transiently expressed MST1, and that protein phosphatase 2A treatment caused a three to fourfold increase in Mst1 kinase activity in vitro, pointing to the presence of an inhibitory phosphorylation (Creasy and Chernoff 1995b). Constitutive activation of PI-3 kinase (Khokhlatchev et al. 2002; Yuan et al. 2010) or AKT (Yuan et al. 2010; Jang et al. 2007) inhibits apoptosis induced by MST1 overexpression as well as MST1 cleavage and nuclear translocation. Moreover, increased activation of endogenous MST1 is evident in AKT1-null as compared with WT MEFs and after RNAi depletion of AKT2 (Yuan et al. 2010). Indeed, AKT was shown to interact with human MST1 and to phosphorylate Threonine 120, a highly conserved AKT phosphorylation consensus motif, both in vitro and in vivo. AKT-mediated phosphorylation of MST1 in response to IGF1 inhibits MST1 cleavage, autophosphorylation, kinase activity, and its nuclear translocation, resulting in the inability of MST1 to induce apoptosis and activate JNK1 and FOXO3a (Yuan et al. 2010).

MST1[T387] (KRRDET^{387}M) as an avid site of AKT phosphorylation in vitro, and this phosphorylation can be stimulated by the treatment of cells with EGF, a response suppressed by inhibitors of PI-3 kinase but not of PKC (Jang et al. 2007). Mutation of this site (MST1[T387E]) results in reduced cleavage in response to H_2O_2, reduced the ability to induce apoptosis and selective impairment in capacity

to phosphorylate FOXO3 at a site required FOXO3 nuclear translocation and proapoptotic activity; the [T387A] mutant exhibited opposite effects. Phosphorylation of the analogous region in MST2 has not been reported. Notably, the enzyme that dephosphorylates AKT[S473] (Warfel and Newton 2012), the protein phosphatase M family member PHLPP, has been found in complex with MST1 in cells, and in vitro catalyzes the dephosphorylation of T387. Overexpression of PHLPP results in modestly increased MST1[T183] phosphorylation, as well as activation of Jnk, p38, and induction of apoptosis. Conversely, co-expression of the kinase-inactive MST1[K59R] mutant partially suppresses the ability of PHLPP to activate the stress kinases. Thus, it is plausible that there is a linked reciprocal regulation of AKT and MST1. While the mechanisms of PHLPP regulation in vivo have not been defined, it is notably that PHLPP contains RA and PH domains and a leucine-rich segment that binds nucleotide-free RAS. Another mechanism for reciprocal regulation was suggested by the identification of AKT and MST1 in a complex in LNCaP prostate cancer cells (Cinar et al. 2007). Full length MST1 as well as the MST1 fragments 1–330 and 331–487 all bind to the C-terminal region of AKT and can inhibit AKT activity when overexpressed; MST2 acts similarly. Overexpression of the Mst1 fragments in zebrafish suppressed the phenotypes elicited by activated AKT. Whether this inhibition reflects simply the ability of an overexpressed AKT substrate to interfere with the binding of other substrates, or a physiologic regulatory mechanism is not known.

Additional studies have shown that IGF1 and AKT inhibit MST2 activity, susceptibility to caspase cleavage and apoptotic efficacy, concomitant with phosphorylation of MST2[T117] (Kim et al. 2010). Consistent with a role for this growth factor signaling pathway in MST regulation, serum withdrawal from NIH3T3 cells appears to promote the activity of a cleaved form of MST2 within 4 h after withdrawal; re-addition of serum reduces kinase activity within 6 h (Wang and Fecteau 2000).

A second, potential mechanism by which AKT-mediated phosphorylation specifically regulates MST2 involves shuttling MST2 between complexing with either RASSF1 or RAF1. In particular, Phospho-MST2[T117] exhibits reduced binding to RASSF1A and increased binding RAF1, thereby promoting MST2 inhibition beyond that achieved by the phosphorylation itself (Romano et al. 2010). The relative importance of this shuttling mechanism as compared with the direct effects of phosphorylation on MST2 catalytic activity is not known.

3.3.2.5 Regulation by the Kinase TAO

Treatment of cells with the protein phosphatase inhibitor okadaic acid results in pronounced activation of endogenous or recombinant MST1 and MST2 (Taylor et al. 1996; Praskova et al. 2004; Creasy et al. 1996). Such activation of the recombinant MST1/MST2 homodimers can be explained by unopposed ongoing transmolecular autophosphorylation, as occurs in vitro by addition of Mg+ATP to recombinant MST1/MST2 (Praskova et al. 2004). Endogenous MST1/MST2

polypeptides, however, are likely bound through their SARAH domain as a monomer to a regulatory protein subunit (SAV1 or an RASSF protein) and/or to a scaffold (e.g., c-RAF) and therefore may be unable to readily autophosphorylate. If so, some additional element is needed for MST1/MST2 activation, either to promote autophosphorylation or to catalyze the activating phosphorylation. The ability to autoactivate does not preclude a role for an upstream activating kinase. In this regard, screens using shRNA-mediated depletion of Drosphila Ste20 kinases (Boggiano et al. 2011) or all 250 protein kinases (Poon et al. 2011) yielded the Serine-Threonine kinase Thousand And One amino acid-1 (TAO) as a negative regulator of Yorkie output. TAO kinases are Ste20p-related MAP kinase kinase kinases (MAP3Ks) that activate p38 MAPK in response to various genotoxic stimuli. It has also been proposed that Drosophila TAO, as well as its three mammalian homologues, to function as a link between the actin and microtubule cytoskeletons, regulating microtubule stability in response to actin signals, by phosphorylation of a variety of microtubule-associated proteins (MAPs) (Timm et al. 2003; Liu et al. 2010; King and Heberlein 2011). Importantly, loss of TAO function in Drosophila increases proliferation in a manner dependent on Hippo, Lats and Yki, and is necessary for the ability of the upstream regulators Merlin (Mer), Expanded (Ex), and Kibra (Kib) to suppress Yki output. Moreover, TAO can directly phosphorylate Hippo on the activation loop, and its mammalian homologue can similarly phosphorylate MST1/MST2 and activate MST2 (see Upstream Signals in Fig. 3.2, also see Fig. 3.3). Regulation of the TAO kinases is as yet poorly defined, and while regulation by the Merlin/Expanded/Kibra complex may occur, the direct association with TAO has yet to be shown. Signals from the actin or microtubular network, likely upstream inputs to the Hippo pathway (Densham et al. 2009; Sansores-Garcia et al. 2011; Zhao et al. 2012), also probably communicate with TAO-1, however the molecular elements linking the cytoskeleton to the Hippo pathway are as yet unknown.

3.3.2.6 Regulation of MST2 by RAF1

The RAF kinases are MAP3Ks which are directly regulated by RAS-GTP, and whose best defined physiological substrates are MAPK1,2, the activators of ERK1,2 (Avruch et al. 2001; Matallanas et al. 2011). Inactivation of RAF1 gene (also called c-RAF) in the mouse results in embryonic lethality due to widespread apoptosis (Wojnowski et al. 1998; Mikula et al. 2001). Knock-in of a mutant RAF1 allele (YY340/341FF) that lacks the ability to phosphorylate and activate MAPK1,2 is nevertheless able to rescue fully from lethality (Huser et al. 2001), indicating that this anti-apoptotic function is independent of RAF1 kinase activity. In this regard, RAF1 was found to co-precipitate with MST2, and thereby antagonize the MST2 kinase through a mechanism independent of RAF1 catalytic activity (O'Neill et al. 2004). The authors of this study propose that RAF1 may act as a scaffold that binds to MST2 SARAH domain to prevent MST2 homodimerization and autoactivation, and recruits protein phosphatase 2A, thereby promoting MST2 inactivation (O'Neill

et al. 2004). In cell culture, overexpression of RASSF1A is reported to disrupt the RAF1/MST2 complex. This may also occur in response to FAS-induced apoptosis, a process which involves RASSF1A/MST2-mediated phosphorylation of YAP, and consequent p73 activation (Matallanas et al. 2007).

Whether the regulation of MST2 by RAF1 is biologically significant in vivo is not clear. Notably, association of Hippo with dRaf has yet to be reported, perhaps reflecting the inability of BRAF (the closest mammalian homologue of dRaf) to bind MST2 (Matallanas et al. 2011). Moreover, no viable MST2 $^{-/-}$; RAF1 $^{-/-}$ offspring were retrieved in multiple litters obtained by intercrossing MST2 $^{-/-}$; RAF1$^{+/-}$ mice (Zhou and Avruch, unpublished). Nevertheless, tissue-specific gene deletion might be better suited for uncovering a functional relationship between MST2 and RAF1. For example, cardiac-specific RAF1 inactivation causes heart disfunction and dilatation, as well as cardiac fibrosis; these phenotypes being reversed by concomitant deletion of ASK1, another protein kinase that has been described to co-precipitate with RAF1 (Chen et al. 2001; Yamaguchi et al. 2004). Similar experiments with RAF1 and MST2 might provide better evidence to support a role for RAF1 as a negative regulator of MST2 activation in vivo.

A recent study has suggested a different functional relationship between RASFF1 and MST2, involving the ability of MST2 to promote RAF1 signaling. EGF-induced ERK activation was reduced upon knockdown of MST2 or LATS1,2 in several cell lines, concomitant with decreased levels of the protein phosphatase 2A catalytic subunit and increased inhibitory RAF1(Ser259) phosphorylation (Kilili and Kyriakis 2010).

3.3.2.7 Regulation of the Hippo Pathway by Cell Polarity

Cell polarity has been shown to be an important regulator of the Hippo pathway in epithelial cells, through the action of three major conserved signaling modules that regulate apical–basal cell polarity. The first of these, the crumbs (Crb) complex, is a tight junction-associated component that localizes to the apical domain, next to adherens junctions (Roh et al. 2003). Crumbs is a large transmembrane protein that forms a complex with the adaptors Stardust (Sdt; PALS1,2 in mammals), PALS1 associated tight junction protein (Patj), MUPP1 (also known as MPDZ), LIN7C, and AMOT (also known as Angiomotin). The second complex, which is also found in the apical region of cells, involves the atypical protein kinase C (aPKC) in association with the Bazooka (Baz, named Par3 in mammals) and Par6 PDZ-binding proteins. Finally, the Scribble polarity module, which is localized to the basolateral region, consists of the Scribble (Scrib), Discs large (Dlg), and Lethal giant larvea (Lgl) scaffold proteins. These three complexes interact for the establishment and maintenance of apicobasal cell polarity as the two apical complexes antagonize the activity of the Scribble module.

Many studies report a preferential localization of the components and regulators of the Hippo pathway at the apical membrane (see Cortical Retention of YAP in Fig. 3.2). Thus, in Drosophila, four apically localized transmembrane proteins have

been shown to regulate the Hippo pathway: the atypical cadherins Fat and Dachsous, the cell adhesion molecule Echinoid (Ed) and Crumbs (Willecke et al. 2006, 2008; Ling et al. 2010; Chen et al. 2010; Yue et al. 2012; Robinson et al. 2010). In addition, the protein Kibra and the FERM-domain containing adaptors Merlin and Expanded, which have been described as potent upstream regulators of the Hippo pathway, also localize to the apical membrane (Zhou and Hanemann 2012; Genevet et al. 2010; Badouel et al. 2009), as does the atypical myosin Dachs, which transduces signals from Fat to Wts (Cho et al. 2006; Mao et al. 2006). Apical localization of the Hippo kinase, the Rassf polypeptides, and Mats has also been reported (Grzeschik et al. 2010; Ho et al. 2010). Likewise, in mammals, MST1/MST2, CRB3, MER, KIBRA, and Angiomotin proteins (AMOT, AMOTL1, AMOTL2) are apically localized, and some directly interact with apical polarity components (Densham et al. 2009; Duning et al. 2008; Cole et al. 2008; Ernkvist et al. 2006). In cell culture, an apically localized fraction of MST1 displays high enzymatic activity, and targeting MST1 to the plasma membrane increases its kinase activity (Praskova et al. 2004; Avruch et al. 2006). Thus, the apical membrane may have properties that increase the activity of Hippo signaling by serving as a site where apical proteins participate as a scaffold in the assembly of the Hippo pathway components.

Along these lines, the Crumbs polarity complex has been shown in mouse to couple cell density sensing to the Hippo pathway by interacting with YAP/TAZ, then facilitating its phosphorylation and its cytoplasmic retention (Varelas et al. 2010). While they prominently localize in the nucleus in cells grown at low or moderate density, YAP/TAZ are sequestrated in the cytoplasm in high-density cultured cells. Affinity purification-mass spectrometry performed on dense cultures identified strong interactions of YAP/TAZ with many components of the Crumbs polarity complex (Varelas et al. 2010). The assembly of the Crumbs complex at high density facilitates YAP/TAZ phosphorylation, leading to its cytoplasmic retention and subsequent suppression of TGFβ-SMAD signaling through YAP/TAZ-mediated sequestration of SMAD. Indeed, knockdown of either CRB3 or PALS component in high cell-density cultures strongly decreases YAP phosphorylation while increasing both YAP/TAZ and SMAD2/3 nuclear localization (Varelas et al. 2010). How Crumbs complex affects the localization of YAP/TAZ is not clearly defined but likely involves AMOT proteins (AMOT, AMOTL1, AMOTL2). These proteins have been described to regulate YAP tight junction localization by direct binding to its WW domain (Zhao et al. 2011). Knockdown of AMOTL2 in polarized Madin-Darby canine kidney (MDCK) cells decreases YAP subcellular localization and leads to YAP activation, as indicated by attenuated YAP phosphorylation, accumulation of nuclear YAP, and induction of YAP target expression. At least in some contexts, AMOT proteins may facilitate MST1/MST2 function, as AMOTL2 knockdown leads to the inhibition of YAP/TAZ phosphorylation, resulting in loss of cell contact inhibition and cell transformation despite the presence of Hippo pathway proteins (Zhao et al. 2011). Although the precise mechanism by which the AMOT proteins control YAP/TAZ phosphorylation remains elusive, one possibility is that AMOT serves as a scaffold to cluster some of the Hippo pathway components at tight junctions to trigger LATS1/LATS2 activation and growth inhibition in response

to increased cell density (Paramasivam et al. 2011). Along these lines, AMOTL2 was shown to directly bind to MST2 and LATS2, and all of the three AMOT members are capable of promoting LATS2 activation, causing a major increase in YAP phosphorylation (Paramasivam et al. 2011).

In addition to the Crumbs complex, the aPKC cell polarity complex and the Scribble module have also been described to regulate Hippo signaling, mainly through genetic studies conducted in *Drosophila* (Grzeschik et al. 2010; Sun and Irvine 2011). Notably, the cell polarity regulators aPKC and Lgl have been proposed to regulate proliferation and survival through the control of the Hippo pathway, by driving Hippo and RASSF sublocalization. Either overexpression of an active form of the Serine-Threonine kinase aPKC or depletion of one of the three components of the Scribble polarity module (i.e., *Scrib*, *lgl*, *or dlg*) in *Drosophila* imaginal discs leads to overgrowth due to increased cell proliferation (Agrawal et al. 1995; Bilder et al. 2000; Grzeschik et al. 2007; Eder et al. 2005; Grifoni et al. 2007). This hyper proliferation is dependent on the activity of Yki, which seems to be activated at least partially through Jun kinase signaling (Grzeschik et al. 2010; Sun and Irvine 2011). Interestingly, Lgl depletion or aPKC overexpression results in mislocalization of Hippo and Rassf proteins from their normal apical localization (where they overlap with aPKC and Dlg) to a more basolateral position (Grzeschik et al. 2010).

Mammalian SCRIB has been proposed to regulate the Hippo pathway through TAZ inhibition in epithelial cells, by serving as an adaptor to assemble a protein complex with TAZ, LATS, and MST at the membrane, and may be required for MST-dependent activation of LATS and ultimately TAZ phosphorylation (Cordenonsi et al. 2011). This mechanism may be highly relevant to cancer as endogenous TAZ was shown to be required for self-renewal and tumor initiation properties in breast cancer stem cells and that TAZ activity is promoted by epithelial-to-mesenchymal transition (EMT), along with dramatic changes in Scribble subcellular localization.

3.3.2.8 Regulation by Adherens Junctions

Adherens junctions (Ajs) are protein complexes in between cells that mediate intercellular adhesion and couple adhesion to the cytoskeleton in order to coordinate the behavior of a population of cells in epithelial tissues (Niessen and Gottardi 2008). Ajs are implicated in the maintenance of the contact inhibition of proliferation process, a well-known property of normal differentiated tissues that allows their proper morphogenesis and architectural maintenance (Fagotto and Gumbiner 1996). Ajs are composed of large transmembrane nectin and cadherin proteins that form trans-homodimers and which intracellular domains link to and interact with signaling pathways through catenins as intermediates (α-catenin, β-catenin, and p120 catenin). Some components of the AJs have been described to interact with the Hippo pathway. Among them, α-catenin was shown to act as an upstream negative regulator of YAP in the epidermis by recruiting phosphorylated YAP through direct binding with the 14-3-3 protein and may, at least partially, explain the already known

tumor suppressor function of α-catenin in the skin (Schlegelmilch et al. 2011; Silvis et al. 2011). By sequestering a pool of YAP to the Ajs, α-catenin may prevent its dephosphorylation by the PP2A phosphatase, and thus its oncogenic activity, as loss of α-catenin results in more efficient association of YAP to PP2A (Schlegelmilch et al. 2011). YAP activation in the skin can lead to the development of squamous cell carcinomas, most likely by promoting the proliferation of epidermal stem and progenitor cells (Schlegelmilch et al. 2011; Silvis et al. 2011).

The role of other components of the Hippo pathway as well as other Ajs proteins during the regulation of YAP is not clear and might be cellular-dependent. The observation that YAP is nonresponsive to depletion of MST1/MST2 or LATS1/MST2 kinases in epidermal cells implies that an undefined mechanism for YAP inactivation, most likely involving another kinase, must predominate in this cell type in order to regulate the localization of phosphorylated-YAP through its association with α-catenin. Moreover, in this cell type, YAP is neither responsive to the depletion of E-cadherin and/or P-cadherin, the usual transmembrane partners of α-catenin, indicating that another membrane receptor mediates YAP inhibition in keratinocytes (Schlegelmilch et al. 2011). These observations contrast with the observation that in breast epithelial cells, E-cadherin seems to act as an adhesion receptor that regulates YAP localization via catenins and the canonical Hippo pathway (Kim et al. 2011). In conclusion, the identification of the Ajs components E-cadherin and α-catenin as regulators of YAP illuminate what could be a central element of the contact inhibition mechanism, in which intercellular adhesion events block proliferation and which disruption is an hallmark of solid tumors (Hanahan and Weinberg 2000; Mayor and Carmona-Fontaine 2010).

3.4 Conclusions

Genetic and functional studies have demonstrated that the Hippo pathway is an evolutionarily conserved program for integrating cell–cell contact with the regulation of cell proliferation, differentiation, and apoptosis. However, in mammals, the pathway shows a complexity unanticipated by the Drosophila genetics. In particular, there is significant tissue-specific diversification of the functions, architecture, and regulation of mammalian Hippo signaling. The upstream inputs regulating MST1/MST2 remain incompletely understood, with uncertainty relating to how the cell–cell contact complexes in polarized epithelia and the internal signals from the actin and microtubular networks coordinately control MST1/MST2 kinase activity. The interplay between these inputs and the function of other signal transduction pathways also requires further elucidation. Scaffold proteins play a critical role both in the regulation of MST1/MST2 activity and in defining its immediate substrates, although the associated mechanisms have yet to be characterized in depth. Finally MST1/MST2 cleavage appears to be associated with high levels of kinase activity some tissues under normal conditions, e.g., normal liver, however the biochemical mechanism and functional significance for this process need to be defined.

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Part II
YAP and TAZ

Chapter 4
YAP1 Uses Its Modular Protein Domains and Conserved Sequence Motifs to Orchestrate Diverse Repertoires of Signaling

Marius Sudol, Irwin H. Gelman, and Jianmin Zhang

Abstract Yes-associated protein 1 (YAP1) is a potent oncogenic protein and is one of the two main effectors of the Hippo tumor suppressor pathway. Originally, YAP1 cDNA was isolated by screening expression libraries for proteins that associate with SH3 domains of Yes and Src protein-tyrosine kinases. Subsequently, YAP1 was shown by homology searches or functional assays to encode multiple protein–protein binding modules including a WW domain, a PDZ domain-binding motif, and TEAD-interaction domain (TID) as well as a transcriptional activation domain (TAD). The TID region encodes a major regulatory phosphorylation site, Serine 127, which plays a critical role in regulating the subcellular localization of YAP1. The TAD region contains a putative coiled-coil region, whose function is unknown, and a tyrosine phosphorylation site that is the subject of intense study. Through reductionistic approaches of molecular and cellular biology, we have gained insight into the detailed function of most of the individual domains, motifs, and selected phosphorylation sites of YAP1. Here, we review how these YAP1 domains act in concert to regulate cell contact inhibition as well as a balance between cell proliferation and apoptosis. Given the mounting evidence that many parameters of malignant cancer progression are driven by Hippo-regulated pathways, understanding the details of how YAP1 regulates signaling is of paramount importance in designing effective strategies to control the oncogenic function of YAP1.

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_4,

Keywords WW domain • mRNA splicing isoforms • Coiled-coil region • Transcriptional activation domain • TEA domain-containing transcription factor interaction domain • PDZ domain

4.1 Introduction

YAP1 (**Y**es-**a**ssociated **p**rotein **1**) is a potent oncogene and is one of the two main effectors of the Hippo tumor suppressor pathway (reviewed by Pan 2010). Originally, YAP1 cDNA was isolated by screening expression libraries for proteins that associate with SH3 (**S**rc **H**omology **3**) domains of Yes and Src protein-tyrosine kinases (reviewed by Hong and Guan 2012). Subsequently, homology searches or functional assays have shown YAP1 to encode multiple protein–protein binding modules including a WW domain (Tryptophan–Tryptophan domain), a PDZ-BM (**P**ost synaptic density-96, **D**iscs Large, **Z**onaula Occludens-1 domain-**b**inding **m**otif), and TID (**T**EA domain-containing factor-**i**nteraction **d**omain), as well as a TAD (**t**ranscriptional **a**ctivation **d**omain) (reviewed by Sudol and Harvey 2010). The TID region encodes a major regulatory phosphorylation site, S127 (Serine 127), which plays a critical role in regulating the subcellular localization of YAP1. The TAD region contains a putative **c**oiled-coil (C) domain, whose function is unknown, and a tyrosine phosphorylation site that is the subject of intense study. Through reductionistic approaches of molecular and cellular biology, we have gained detailed insight into the detailed function of most of the individual domains, motifs, and selected phosphorylation sites of YAP1. In this chapter, we review how these YAP1 domains act in concert to regulate cell contact inhibition as well as a balance between cell proliferation and apoptosis. Given the mounting evidence that many parameters of malignant cancer progression are driven by Hippo-regulated pathways, understanding the details of how YAP1 regulates signaling is of paramount importance in designing effective strategies to control the oncogenic function of YAP1.

4.2 YAP1 Discovery and Its Modular Structure

YAP1 was first identified by virtue of its ability to associate with the SH3 domain of Yes and Src protein-tyrosine kinases (Sudol 1994). The chicken YAP1 gene was shown to encode a protein of 65 kDa that was easily precipitated from normal chicken embryo fibroblasts as a phospho-serine-rich protein. No traces of threonine or tyrosine phosphorylation were detected in the phospho-amino acid analysis of the precipitated YAP1. A PxxP (P—Proline, x—any amino acid) consensus motif for binding to SH3 domains was found in the middle of the YAP1 molecule and it was confirmed as a functional motif that is involved in YAP1-Yes kinase interaction

in in vitro binding assays (Sudol 1994). When the mouse and human orthologs of the YAP1 gene were cloned, a repeated and inserted block of semi-conserved 38 amino acids was identified within the amino terminal half in the mouse YAP1 protein (Bork and Sudol 1994; Sudol et al. 1995). Since this repeated block also showed significant sequence similarity to repeats in other proteins, including that of human Dystrophin and yeast Rsp5 E3 ubiquitin ligase, it was suspected that it might represent a modular protein domain, such as the SH2 and SH3 domains (Bork and Sudol 1994; Pawson 2004). Computer-aided homology searches of all known protein sequences confirmed the suspicion, and this block was provisionally named the WW domain, after the presence of two conserved tryptophans spaced 20–21 amino acids apart (Bork and Sudol 1994; Sudol et al. 1995). Shortly after the initial publication, two other groups also reported the same domain as the WWP motif (Andre and Springael 1994) and the Rsp5 repeat (Hofmann and Bucher 1995). The WW domain became a *bona fide* modular protein domain when it was shown that it mediated protein–protein interactions with ligands containing Proline-Proline-any-amino-acid-Tyrosine (PPxY) consensus motifs (Chen and Sudol 1995) and when the structure of the domain in complex with its ligand peptide was elucidated (Macias et al. 1996).

Apart from the WW domain, the modular structure of YAP1 contains a Proline-rich region (PR) at the very amino terminus, which is followed by a TID region. Next, following a single WW domain, which is present in YAP1-1 isoform, and two WW domains, which are present in the YAP1-2 isoform, there is the SH3-BM (SH3 binding motif) (Ren et al. 1993) (Fig. 4.1b). Following the SH3-BM is a TAD region which contains a coiled-coil (**C**) domain and a PDZ binding motif (PDZ-BM) (Fig. 4.1b). The two major isoforms of human YAP1, namely YAP1-1 and YAP1-2, are generated by differential mRNA splicing of exon 4, which encodes the second WW domain (Fig. 4.1b) (Sudol et al. 1995).

Below, we will briefly discuss the individual domains and regions of YAP1 protein, describing in detail how they were originally identified and what is known about their functions. We will try to present an integrated picture of how they function in concert to regulate cell contact inhibition as well as the fine balance between proliferation and apoptosis. In particular, we will emphasize the versatility and plasticity of YAP1 WW domains.

4.3 The Role of TEAD Interacting Domain, TID

The family of four transcription factors known as TEAD factors was shown to require YAP1 for its transcriptional activity (Vassilev et al. 2001). At the amino terminal region of TEADs resides a TEA domain that recognizes GGAATG elements in the promoter region of target genes (Anbanandam et al. 2006). Many of these genes robustly regulate cell proliferation, such as *AXL* and CTGF, or inhibit apoptosis, such as *BIRC5* (Zhao et al. 2009; Zhang et al. 2008). The protein

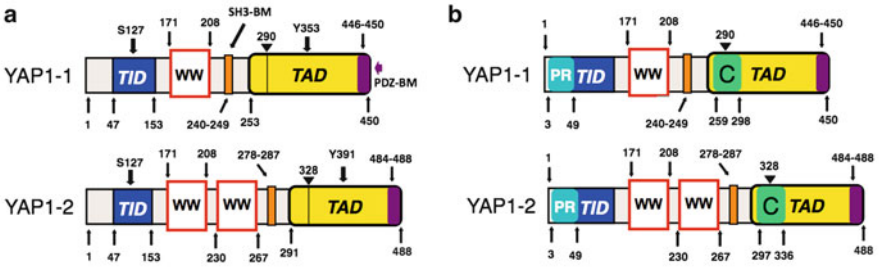

Fig. 4.1 (**a**) Schematic structure of YAP1 isoforms depicting functional regions, modular domains, domain binding motifs, and major serine and tyrosine phosphorylation sites. At the amino terminal region of YAP1, there is a TEAD interaction domain (TID), shown in *blue*, which was originally delineated for the mouse YAP1 protein in an in vitro protein-binding assay. This arbitrarily chosen region of YAP1 amino terminus corresponds to amino acids 32–139 in mouse YAP1 and to residues 47–153 in human YAP1. Within the TID domain, there is a major Serine phosphorylation site S127 (*black arrow*) that functions as 14-3-3 protein-binding site. YAP1-1 and YAP1-2 isoforms differ by the number of WW domains (depicted as *red boxes*). The length of the WW domain of YAP1 is estimated as 38 amino acids based on the degree of sequence similarity among proteins containing WW sequences. Also, the length of the WW domain was discerned from the differentially spliced exon 4 in human YAP1-2 isoform, which encodes the second WW domain and is 38 amino acids a long (Bork and Sudol 1994). The PxxP consensus motif for SH3 domain binding PVKQPPPLAP is located between WW domain and TAD domain and indicated as *orange rectangle*. TAD domain (shown in *yellow*) was delineated in a GAL4-based transactivation assay using a mouse YAP1 carboxy-terminal fragment encompassing amino acids 276–472, where 472 L residue is the last coding amino acid of the mouse YAP1 isoform. This arbitrarily chosen region of YAP1 carboxy-teminus is termed TAD domain and corresponds to residues 253–450 in human YAP1-1 and to residues 291–488 in human YAP1-2. Within the TAD domain at position 290 in YAP1-1 and position 328 in YAP1-2, there is a region where additional sequences are spliced in various isoforms of YAP1 gene. YAP1 is tyrosine phosphorylated on Y357 in one of the YAP1-1 isoforms within TAD that contained a spliced in sequence at position 290. In the isoforms YAP1-1 and YAP1-2 without splicing in the TAD region, the Y357 site (SS**Y**SVPRT) corresponds to Y353 and Y391, respectively. The last 5 carboxy-terminal residues—FLTWL (shown in *dark violet*) represent PDZ domain binding motif. (**b**) Schematic structure of YAP1 isoforms depicting two additional regions whose function is unknown. At the amino terminal region, YAP1 harbors Proline-rich region (PR) between amino acids 3–49, shown in *light blue*. Within the TAD domain there is a coiled-coil domain (C), shown in *light green*, which was predicted byCoils 2 program available via SMART resource as the following sequence: GSNSNQQQQMRLQQLQMEKERLRLKQQELLRQELALRSQL that is located at position 259–298 in YAP1-1 and 297–336 in YAP1-2. Curiously, the C domain covers the 290 and 328 positions, which are sites of spliced in sequences in some of the YAP1 isoforms. These spliced in sequences may disrupt the C domain structure and function

complex between YAP1 and TEAD2 factor was originally characterized by the DePamhilis laboratory at the NIH (Vassilev et al. 2001). In their detailed study, the amino-terminal region of YAP1, containing the TID region, was shown to interact physically and functionally with the carboxyl-terminal region of TEAD2. This finding had important ramifications for the emerging Hippo pathway because it connected cytoplasmic signals of serine-threonine kinases and its effector YAP1 with a transcriptional program. Specifically, the Drosophila ortholog of mammalian TEAD,

Scalloped, was quickly shown as a major transcription factor for the Drosophila ortholog of YAP1, Yorkie (Yki) (Zhang et al. 2008). The growth-promoting and oncogenic activities of YAP1 and Yki were shown to require complex formation with TEAD and Scalloped, respectively.

The structure of YAP1 in complex with TEAD factor was solved at high resolution and serves as a subject of intense structure–function analyses and drug discovery efforts (reviewed by Sudol et al. 2012). The disruption of this complex by small molecules should help in controlling cancers that harbor amplification of the YAP1 gene, such as hepatocellular carcinomas. The TID is composed of two α helices and a hydrophobic linker, which together form a clip-like structure that is accommodated by three major sites of interaction on TEAD protein (Chen et al. 2010; Li et al. 2010; Tian et al. 2010). The interface between TID and TEADs is druggable and a recent report provides a successful example of a small molecule inhibitor that attenuates the YAP1-TEAD complex (Liu-Chittenden et al. 2012).

4.3.1 Serine 127 as a Critical Regulatory Site

Intriguingly, within the TID domain there is a major site of regulatory phosphorylation at S127 (Fig. 4.1b), which was first mapped by the Downward laboratory (Basu et al. 2003) in a screen for B/AKT protein kinase substrates that associate with 14-3-3 proteins when phosphorylated. Although the AKT kinase is not the major kinase that phosphorylates YAP1 on S127, and the pro-apoptotic function of YAP1 used as a biological "read-out" by the team of Julian Downward is observed for YAP1 only under conditions of stress, this study was critical in showing that a single site of serine phosphorylation can promote the cytoplasmic localization of YAP1 via 14-3-3 proteins, and therefore dramatically curtail its function as a nuclear transcriptional co-activator. There are no solid reports on S127 phosphorylated YAP1 being detected in the nucleus; however, if the phospho-S127-YAP1 would find its way to the nuclear compartment, it would not be able to form TEAD/YAP1 transcriptional complex because of steric hindrance.

4.3.1.1 WW Domain Partners Play Diverse Roles

The Hippo tumor suppressor pathway is highly enriched with proteins that cross talk via their WW domains and PPxY motif ligands (Sudol and Harvey 2010). None of the other signaling pathways that we know at present utilize WW domain complexes to the extent seen in the Hippo pathway.

The protein partners of the YAP1 WW domains, which are part of the Hippo signaling pathway or which network from cross talking pathways can be divided into two groups. One group is represented by LATS (large tumor suppressor) kinases, members of the AMOT (Angiomotin) family and PTPN14 (protein-tyrosine phosphatase of non-receptor type number 14), which act to retain YAP1 in the

cytoplasm (Hao et al. 2008; Oka et al. 2008, 2012; Chan et al. 2011; Zhao et al. 2011; Wang et al. 2011; Huang et al. 2012; Liu et al. 2012). The other group of WW domain ligands is represented by transcription factors, such as the RUNX (Runt domain-containing proteins) family of transcription factors, p73 pro-apoptotic factor and the SMAD (SMA—after small protein, and MAD—after mothers against decapentaplegic protein) factors (Yagi et al. 1999; Strano et al. 2001; Ferrigno et al. 2002). In addition, a transcriptional regulator WBP2 (WW domain binding protein 2) also binds to YAP1 WW domains (Chen et al. 1997) to regulate gene expression in a phosphorylation-dependent manner (see below). Although most of the ligands of YAP1 WW domains contain the PPxY motif, some of them employ additional noncanonical sequences for efficient complex formation, such as those used by certain SMADs (Massague 1998; Mauviel et al. 2012). A very interesting study, which illuminated the versatility of YAP1 WW domains, was recently completed by the Macias and Massague laboratories (Alarcon et al. 2009; Aragon et al. 2011). They showed that activation of TGB β/BMP (transforming growth factor b/bone morphogenic proteins) pathway induced SMAD1 binding to the YAP1-WW domain via its PPxY motif and an additional phosphorylated linker sequence, resulting in translocation to the nucleus and the regulation of target genes. The phosphorylated linker of SMAD1 is located in close proximity to the PPxY motif, and two CDKs (cyclin-dependent kinases), CDK8, which curiously harbors a conserved PPxY motif as well, and CDK9, which have been implicated as potential linker kinases (Alarcon et al. 2009; Aragon et al. 2011). In contrast, the complex of YAP1 with inhibitory SMADs, such as SMAD7, seems to be constitutive and could be phosphorylation-independent (Ferrigno et al. 2002). The ability of YAP1 WW domains to recognize phosphorylated and non-PPxY-containing motifs indicates the tremendous plasticity of the WW module in reaching a variety of partners to orchestrate diverse signaling events.

The YAP1-1 and YAP1-2 isoforms, which differ in their number of WW domains seem to signal differently. For example, YAP1-1 does not bind p73 factor and cannot induce apoptosis when HEK293 cells are stressed by low serum conditions (Oka et al. 2008). Moreover, YAP1-1 does not interact with Angiomotin-Like-1 (AMOTL1) protein while YAP1-2 does. Also AMOTL1 has the ability to inhibit YAP1-2 function by preventing its nuclear localization (Oka et al. 2012). More work needs to be done to characterize the differences between YAP1-1 and YAP1-2 isoforms in order to address a potential dominant negative activity of YAP1-1.

4.4 SH3 Binding Motif

The SH3 BM of YAP1 was shown to be required for YAP1–Yes-SH3 domain complex formation in in vitro binding assays. A 22-amino acid peptide, which spanned the PxxP motif of YAP1, was shown to compete with YAP1 binding to purified Yes-SH3 fragments. Little has been done on the SH3 BM because initially YAP1 could not be shown to be tyrosine phosphorylated in cells grown under standard

conditions. However, the Ito laboratory reported that YAP1 deleted of its SH3 BM was less active in GAL4-based transcription activation assay (Yagi et al. 1999), suggesting that SH3 domain-containing proteins could modulate the transcriptional function of YAP1. In addition, a recent report shows that YAP1 recruits the c-Abl kinase to phosphorylate the Nedd4-2 ubiquitin ligase, suggesting that the SH3 BM of YAP1 might be involved in this process (Skouloudaki and Walz 2012). With several recent reports of YAP1 being phosphorylated by non-receptor protein-tyrosine kinases, and interacting with a protein-tyrosine phosphatase (see below), it is expected that the role of the SH3 BM in the function of YAP1 will be revisited.

4.5 Transcription Activation Domain, TAD

The Ito laboratory was the first to show that YAP1 is a strong transcriptional co-activator, rivaling even herpes simplex virus VP16 known for its promiscuous transactivation ability (Yagi et al. 1999). YAP1-2 employs its WW domains to recognize and form a stable complex with PPxY motif in Polyoma enhancer binding protein 2 α (PEBP2α), one of the RUNX family transcription factors, which regulates transcription of the osteocalcin gene. Subsequent studies that characterized YAP1 complexes with p53 binding protein 2 (p53 BP2) and the carboxy-terminal fragment of ErbB4 also revealed that YAP1 has the ability to act as a transcriptional co-activator (Espanel and Sudol 2001; Komuro et al. 2003). The TAD of YAP1 was mapped within the carboxy-terminal half of the YAP1 protein (see Fig. 4.1b) (Yagi et al. 1999). It is important to note that the boundaries of TAD in YAP1 were chosen arbitrarily and that the actual functional region of TAD may be shorter than the one that was originally delineated. Within the TAD region, there are three important elements: the C domain, a Tyrosine 357 (Y357) phosphorylation site and PDZ-BM.

4.5.1 Coiled-Coil Domain

Even though the C domain of YAP1 was identified a decade ago, its function remains unknown (Mohler et al. 1999). It is likely that through the C domain, YAP1 forms complexes with other proteins, especially nuclear proteins, which have cognate coiled-coil regions. Perhaps via such complexes, the transactivation function of YAP1 could be finely regulated. One of the potential candidates for the coiled-coil-mediated interaction with YAP1 is c-Jun that harbors a coiled-coil domain itself and also has a conserved PPxY motif for additional contact with YAP1 via the WW domain (Einbond and Sudol 1996; Kanai et al. 2000; Hu and Li 2002; Gao et al. 2006). The proposal of the existence of YAP1-c-Jun complex is supported by a short report suggesting that some of the functions of YAP1 are indeed c-Jun-dependent (Danovi et al. 2008). There could also be cytoplasmic proteins that contain coiled-coil domains and interact with the C domain of YAP1 to modulate its activity (see below).

Curiously, several YAP1 isoforms, which have been characterized so far (Sudol et al. 1995; Komuro et al. 2003; Muramatsu et al. 2011) contain spliced insertion sequences within the C domain of YAP1. We suggest it will be interesting to explore these isoforms to see if their cytoplasmic and nuclear functions differ from the isoforms with intact C domains.

4.5.2 Tyrosine 357 Site

Originally, three publications raised the possibility that YAP1 could be phosphorylated on tyrosine in some signaling scenarios (Zaidi et al. 2004; Levy et al. 2008; Tamm et al. 2011). The Stein laboratory reported that YAP1 tyrosine phosphorylation is necessary for its interaction with Runx2 transcription factor as well as for its subsequent nuclear trafficking (Zaidi et al. 2004). Moreover, inhibition of the Src and Yes kinase activity reduced the tyrosine phosphorylation of YAP1, resulting in the dissociation of the endogenous Runx2-YAP1 complexes (Zaidi et al. 2004). A second publication from the Shaul laboratory reported that in response to DNA damage, YAP1 is phosphorylated by c-Abl kinase on Y357 and this modification stabilizes YAP1 protein. Furthermore, p-Y357 YAP1 has higher affinity for the p73 transcription factor and this association results in increased expression of p73-dependent and pro-apoptotic target genes (Levy et al. 2008). The third publication came from the Anneren laboratory reporting that Yes kinase binds and phosphorylates YAP1 and activates the YAP1-TEAD2-dependent transcriptional program underlying the self-renewal process of mouse embryonic stem cells (Tamm et al. 2011).

Phosphorylation of YAP1 on tyrosine has become a subject of intense investigation. Recently, a novel regulatory mechanism for the YAP1 oncogenic function by PTPN14 was reported (Huang et al. 2012; Liu et al. 2012). YAP1 and PTPN14 form a complex through direct interaction between the PTPN14 PPxY motifs and YAP1 WW domains (Huang et al. 2012; Liu et al. 2012). PTPN14 contains two conserved structural elements: an amino terminal FERM domain (band 4.1-ezrin-radixin-moesin family of adhesion molecules) and a carboxy-terminal PTP (protein-tyrosine phosphatase) domain. The subcellular localization of the PTPN14 protein depends on a variety of factors, such as cell type, cell-matrix adhesion status (Ogata et al. 1999a), presence of serine phosphorylation (Ogata et al. 1999b), and cell confluence (Wadham et al. 2000; Wyatt and Khew-Goodall 2008). YAP1 is a direct substrate of PTPN14, and PTPN14 inhibits the transcriptional co-activator function of YAP1 by retaining it in the cytoplasm. As expected, the knockdown of PTPN14 leads to nuclear retention of YAP1 as well as to an increase in the YAP1-dependent cell migration (Liu et al. 2012). This finding is in agreement with a previous publication reporting a potential tumor-suppressive function of PTPN14 in colon cancer (Wang et al. 2004). Taken together, these data revealed a new mechanism by which the function of YAP1 might be regulated.

In addition to YAP1, PTPN14 is reported to mediate the dephosphorylation of tyrosine residues in an adherens junction protein, β-catenin (Smith et al. 1995; Wadham et al. 2003). Also, it has been recently shown that YAP1 interacts with adherens junction protein α-catenin in the epidermis (Schlegelmilch et al. 2011; Silvis et al. 2011). Binding of the YAP1/14-3-3 complex to α-catenin stabilizes this complex and inhibits the access of PP2A to YAP1 (Schlegelmilch et al. 2011). Furthermore, the Crumbs polarity complex, which includes PALS1, PATJ, MUPP1, LIN7C, and AMOT proteins, interacts with YAP1 and relays cell density information by promoting YAP1 phosphorylation and its cytoplasmic retention, as well as by suppressing the TGF-β signaling (Varelas et al. 2010). These data strongly suggest that there must be extensive cross talk among adherens junction and polarity complex proteins that involve PTPN14 and YAP1 (Mauviel et al. 2012). Intriguingly, a recent report from the Stocker laboratory (Poernbacher et al. 2012) demonstrated that *Drosophila* Pez (the ortholog of PTPN14) interacts with KIBRA, a WW domain-containing upstream component of the Hippo signaling pathway (Kremerskothen et al. 2003), and functions as a negative upstream regulator of Yki (the fly ortholog of YAP1) in the regulation of *Drosophila* intestinal stem cell proliferation. Therefore, it is tempting to predict that PTPN14 not only directly interacts with and regulates YAP1 function, but may also regulate YAP1 by indirect interaction with KIBRA.

Although YAP1 was first identified as a Src and Yes kinase-associated protein (Sudol 1994), it is only recently that evidence exists that Src family kinases can phosphorylate YAP1 on Y375 within the TAD region (Huang et al. 2012; Liu et al. 2010; Levy et al. 2008).

4.5.3 PDZ Binding Motif

Screening of random peptide libraries with various PDZ domains resulted in the identification of a number of PDZ ligand sequences including peptides that terminated with the amino acid sequence TWL, the very sequence of the YAP1 carboxy-terminus (Wang et al. 1998). Aided with this data, the Milgram laboratory documented that YAP1 contains a functional PDZ-BM and forms complexes with a PDZ domain of SLC9A3R1 (solute carrier family 9, subfamily A member 3 regulator 1) protein (Mohler et al. 1999). At present, we know of another protein, ZO2 (Zona Occludens 2), that uses its first PDZ domain to form functional complexes with YAP1 and is critical in regulating nuclear localization of YAP1 (Oka and Sudol 2009; Oka et al. 2010). ZO2, like YAP1, has a propensity to shuttle between the cytoplasm and nucleus in a cell density-dependent manner (Balda et al. 2003) and has been shown to regulate nuclear transport of another protein LASP-1 (LIM and SH3 domain protein 1) via its SH3 domain (Mihlan et al. 2012). A YAP1 deletion mutant lacking the FLTWL motif in the PDZ-BM is unable to enter the cell nucleus, implicating PDZ domain-containing proteins in controlling this critical regulatory function.

4.6 Examples of Concerted Actions

The Hippo pathway plays a critical role in regulating cell contact inhibition. (Hong and Guan 2012). The subcellular localization of YAP1 is cell density-dependent. In sparsely populated cultures, YAP1 tends to accumulate in the nucleus and promotes cell proliferation. Whereas in densely populated cultures, YAP1 localizes in the cytoplasm, rendering it incapable of directly regulating transcription. The modular domains and sequence motifs of YAP1 drive an assembly of multicomponent protein complexes, which differ between growing or contact-inhibited cells.

In high-density cultures, YAP1 forms signaling complexes with several cytoplasmic proteins, which apart from their enzymatic or other functions, have the ability to physically sequester YAP1 in the cytoplasm (Fig. 4.2a). Examples of such proteins are the WW domain-interacting LATS kinases, PTPN14 phosphatase and three members of the AMOT family of tight junction proteins (AMOT, AMOT-Like-1, and AMOT-Like-2) (Hao et al. 2008; Oka et al. 2008, 2012; Chan et al. 2011a; Zhao et al. 2011; Huang et al. 2012; Liu et al. 2012). In addition, another tight junction protein, ZO2, uses its first PDZ domain to complex with YAP1 via its PDZ-BM (Oka et al. 2010). As ZO2 and ZO1 are able to heterodimerize, it is expected that at least a subpopulation of YAP1-Angiomotin-ZO2-ZO1 tetrapartite protein complexes localize to tight junctions, not only as sensors of cell-to-cell contacts but also as anchors that prevent YAP1 from nuclear entry.

Although little is known about cytoplasmic proteins that interact with the C domain of YAP1, we speculate here that ROCK1 (**R**ho-associated, **c**oiled-coil domain-containing protein **k**inase **1**) together with Rho GTPase regulates YAP1 through mechano-sensing signals, and may also function as a cytoplasmic anchor (Dupont et al. 2011). Moreover, in epidermal cells, an adherens junction protein, α-catenin, facilitates the interaction of S127 phosphorylated YAP1 with 14-3-3 proteins, preventing access of PP2A to dephosphorylate YAP1, and also further stabilizing the cytoplasmic localization of YAP1 (Schlegelmilch et al. 2011).

Several regulatory loops of positive feedback that further promote cytoplasmic localization of the YAP1 protein and enhance the maintenance of tight junctions were uncovered recently. For example, AMOTL2 was shown to activate LATS2 kinase activity, thereby enhancing the phosphorylation of YAP1 on the regulatory S127 site, promoting its cytoplasmic localization (Paramasivam et al. 2011). Also, YAP1 was recently shown to protect AMOTL1 from Nedd-4.2 E3 ubiquitin-ligase-mediated-degradation by recruiting c-Abl kinase, which phosphorylates Nedd-4.2 on tyrosine residues, thereby negatively regulating its ligase activity (Skouloudaki and Walz 2012). These two examples of concerted action of YAP1 and its WW domain-interacting partners, AMOTL1, AMOTL2, and LATS2, reveal subtle molecular mechanisms that prevent YAP1 from nuclear entry while at the same time ensuring the integrity of tight junctions in densely populated cells (Fig. 4.2a).

In low-density cultures, YAP1 is dephosphorylated on S127 by the PP2A phosphatase and it subsequently translocates into the nucleus in complex with the ZO2 protein, which acts as a nuclear shuttle (Fig. 4.2b, c). The degradation of AMOTs

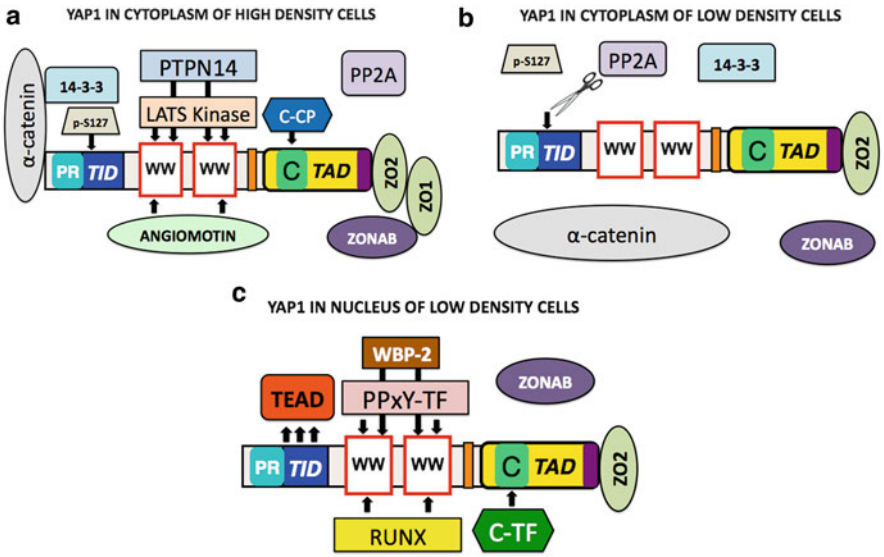

Fig. 4.2 Three scenarios of YAP1 signaling via protein complexes: (**a**) In high-density cells, YAP1 is localized in the cytoplasm and forms complexes with selected cellular proteins. These complexes tend to sequester YAP1 in the cytoplasm. Activation of MST and LATS kinases renders YAP1 phosphorylated on S127, which creates a binding site for 14-3-3 protein that anchors YAP1 in the cytoplasm. In epidermal cells, an adherens junction protein, α-catenin interacts with YAP1-14-3-3 complex, blocking PP2A access to dephosphorylate YAP1 on Serine 127, further stabilizing the complex. The interaction of WW domains with LATS kinases, PTPN14 phosphatase, and Angiomotin also tends to sequester YAP1 protein in the cytoplasm. The C domain of YAP1 may very well interact with cytoplasmic proteins that contain coiled-coil domain (CYT-CP). One potential candidate is ROCK1 (Rho-associated, protein kinase 1), which together with Rho-GTP-ase may mediate mechano-sensing signals to YAP1, independently from the canonical Hippo cassette (Dupont et al. 2011). ZO2 is known to bind to PDZ-BM of YAP1 and since ZO2 heterodimerizes with ZO1, they may also prevent YAP1 from nuclear localization. (**b**) In low-density cells YAP1 is dephosphorylated on S127 by PP2A phosphatase and it is ready to be translocated to the nucleus in complex with the ZO2 protein. In epidermal cells, α-catenin can no longer maintain a complex with YAP1 and 14-3-3 to retain YAP1 in the cytoplasm. (**c**) YAP1 in the nucleus forms complexes with various transcription factors to regulate diverse transcriptional programs. Primarily it interacts with TEAD transcription factors to induce proliferative and anti-apoptotic genes. The WW domains of YAP1 will bind a number of transcription factors that contain PPxY motif (PPxY-TF) such as members of the RUNX family. In addition, WBP2 factor that is tyrosine phophorylated by c-Src and c-Yes kinases positively regulates YAP-1-TEAD complex in promoting cell proliferation. The C domain may act in concert with WW domains of YAP1 and interact with coiled-coil domains of transcription factors (C-TF) such as c-Jun to regulate proliferation. The role of ZO2 and ZONAB in Hippo signaling is still not clear but theoretically intriguing (see text)

by WW domain-containing E3 ubiquitin ligases may release YAP1 from tight junctions. In epidermal cells, α-catenin can no longer maintain a complex with YAP1 and 14-3-3 to retain YAP1 in the cytoplasm. In the nucleus, YAP1 forms complexes with various transcription factors to regulate diverse transcriptional programs. Nuclear YAP1 primarily interacts with TEAD transcription factors to induce both proliferative and anti-apoptotic genes. In addition to the YAP1-TEAD complex that engages the TID domain of YAP1, intact WW domains and TAD region are required to act in unison for YAP1 to mediate proliferation. The WW domains of YAP1 will assemble a number of PPxY motif-containing transcription factors (PPxY-TF) such as members of the RUNX family. In addition, WBP2, which is tyrosine phosphorylated by Src and Yes kinases, positively regulates the YAP-1-TEAD complex and enhances cell proliferation (Chen et al. 1997; Lim et al. 2011). The C domain of YAP1 may act in concert with its WW domains in order to interact with coiled-coil domain-containing transcription factors (C-TF) such as c-Jun to regulate proliferation. We also speculate that as the half-life of ZO-1 decreases in sparsely populated cells and the expression of its cognate transcription factor ZONAB (ZO-1-associated nucleic acid binding protein) increases (Balda et al. 2003), ZONAB will no longer be retained by ZO1 in tight junctions, but will preferentially localize in the nucleus to drive expression of proliferative genes as a Y-box transcription factor.

4.7 Hippo in the Context of Other Cancer Pathways

There is growing evidence that dysregulation of the Hippo pathway may contribute to other pro-oncogenic pathways. For example, a recent genetic link between the Hippo pathway and genes that control apico–basal polarity of epithelial cells, such as Scribble, DLG (Discs LarGe), and LLGL (Lethal Giant Large) (Enamoto and Igaki 2011) suggests that the loss of Hippo mediators contributes to epithelial-to-mesenchymal transition (EMT), a marker of cancer progression (Hanahan and Weinberg 2011). Similarly, the PAR family of polarity-regulating proteins is linked to Hippo pathway regulation (McCaffrey and Macara 2011).

Given that elevated YAP1 expression correlates with oncogenic progression in several human cancers, it is interesting to note that YAP1 expression can be regulated by miR-375, which itself is significantly downregulated in liver cancer (Liu et al. 2010).

The Hippo pathway may also promote oncogenic progression by controlling the differential expression of pro-oncogenic genes. For example, the YAP1 paralog, TAZ, promotes taxol resistance in breast cancer cells by inducing the expression of Cyr61 and CTGF (Lai et al. 2011). Indeed, the anticancer effect of the drug, α-tocopheryl succinate, is due to the suppression of Hippo factors that normally, in complex with Foxo-family transcription factors, repress the expression of pro-apoptosis factors such as Noxa (Valis et al. 2011). A recent report (Xu et al. 2010) suggests that resistance of glioblastoma cells to cytotoxic chemotherapy-induced cell stress

pathways requires the suppression of Hippo signaling. It is interesting to speculate that Hippo pathway components such as LATS1 may normally regulate genotoxic responses by radical oxygen species (ROS) or DNA-damaging agents. This would correlate with the loss of LATS1 expression following oncogene-induced ROS (Takahashi et al. 2006) and the finding that LATS1-deficient cells suffer from premature senescence due to the inability to resolve their cytokinesis defects (Yang et al. 2004). Interestingly, RASSF1A (Ras Association Domain Family 1A) physically interacts with Hippo/MST2 and LATS1, promoting their phosphorylation, and cells deficient in RASSF1A suffer from cytokinesis defects (Guo et al. 2007).

The canonical Wnt/ß-catenin signaling pathway is a critical regulator of cellular proliferation and its cross talks with the Hippo pathway (Clevers and Nusse 2012; Varelas and Wrana 2012). When the Wnt pathway is engaged, the ß-catenin transcription co-activator translocates from the cytoplasm into the nucleus. Nuclear ß-catenin associates with members of the T-cell factor/Lymphoid enhancer factor (TCF/Lef) family of transcription factors and together, ß-catenin/TCF complexes drive expression of growth-promoting genes. Mutations in components of the Wnt/ß-catenin signaling pathway are found in approximately 90 % of colorectal cancers and these mutations contribute to aberrant growth. While the Hippo pathway inhibits Wnt signaling in primary cardiomyocytes and in HEK293 cells (Heallen et al. 2011; Imajo et al. 2012), in colorectal cancer cells the relationship between these two pathways is different. An elegant study by Joe Avruch and colleagues found that deletion of Mst1/Mst2 kinases in the intestinal epithelium lead to accumulation of nuclear YAP1 and activation of ß-catenin signaling (Zhou et al. 2011). Another study demonstrated that ß-catenin/TCF complexes directly activate expression of the YAP1 gene in human colorectal cancer cells and that YAP1 was required for oncogenic properties of these cells (Konsavage et al. 2012). Together, these findings suggest that Hippo and Wnt/ß-catenin signaling pathways may act in concert to drive colorectal carcinogenesis. It is tempting to speculate that perhaps YAP1/TEAD and ß-catenin/TCF transcription complexes converge to activate a shared set of target genes. In support of this hypothesis, a search of 2,168 ß-catenin binding regions identified in a ChIP-Seq screen found that 397 contained coupled TEAD and TCF consensus DNA binding motifs (Greg Yochum, personal communication and Bottomly et al. 2010). Further experiments are required to determine whether all or a subset of these targets are controlled by Hippo and Wnt signaling and whether these targets could be exploited for diagnostic purposes.

4.8 Concluding Remarks

For clarity, we focused our discussion here almost exclusively on YAP1, one of the two main effectors of the Hippo tumor suppressor pathway, and omitted TAZ (WWTR1) which is a close paralog of YAP1. Many features discussed here for YAP1 are also relevant for TAZ; however, subtle structural and functional differences exist between these effectors and are being unraveled at a fast pace now.

We *deconstructed* YAP1 by dissecting its individual modular protein domains, and conserved binding motifs, which are the basic units of the canonical code of signaling. These modules are frequently called the Lego® blocks of Nature because they form a plethora of protein-to-protein complexes in a reiterated and combinatorial fashion, similar to a structure made of interconnecting Lego® blocks (Pawson 2004; Sudol 2004). The wide occurrence of these modules in YAP1, TAZ, and in other proteins of the Hippo network has facilitated a fast dissection of their function and their protein partners. These modules have helped the characterization of signaling steps that link cell density and junctional complexes to transcriptional programs. However, unlike Lego® blocks, modular protein domains and conserved motifs are embedded within host proteins and work in concert to transmit discrete signals. Several examples of this concerted action were discussed here to illuminate the intricacies of these processes. It seems that many parallel signals and positive feedback loops of regulation are acting together in a redundant fashion to maintain a specific state, such as contact-inhibited growth or vigorous proliferation under conditions of subconfluency. We hope that our discussion of YAP1 signaling via modules, motifs, and post-synthetic modifications provides insight into the vast repertoire of signaling processes that are used by the Hippo pathway.

The Hippo tumor suppressor pathway has quickly emerged in the past several years as a new signaling pathway that is directly relevant to human cancer. As discussed above, the pathway cross talks extensively with other pathways (Mauviel et al. 2011; Varelas and Wrana 2012) or with major cancer genes (Aylon et al. 2006, 2010), and understanding the details of signaling by both the canonical Hippo pathway and the extended Hippo network will be of paramount importance in designing new and effective strategies to control cancer.

Acknowledgments We thank our colleagues Virginia Mazack and Wannian Yang for valuable comments on the first version of the manuscript and Gregory Yochum for valuable discussions, comments, and for sharing his unpublished data. This work was supported by PA Breast Cancer Coalition Grants (#60707 an #920093) plus the Geisinger Clinic (to MS), by funds from the National Institutes of Health (Grants# R01-CA94108 and P30-CA016056) and the Department of Defense (PC074228, PC101210) (to IHG), and by Roswell Park Cancer Institute and NCI grant #P30 CA016056 (to JZ).

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Chapter 5
Regulation of YAP and TAZ Transcription Co-activators

Fa-Xing Yu, Bin Zhao, and Kun-Liang Guan

Abstract The Yes-associated protein (YAP) and WW domain-containing transcription regulator 1 (WWTR1, also known as TAZ) are two transcription co-activators that act downstream of the Hippo tumor suppressor pathway. YAP/TAZ regulate expression of a large number of genes that are important in controlling organ size, tumorigenesis, and stem cell functions. The activity of YAP/TAZ is mainly inhibited by Lats kinases of the Hippo pathway. Upon phosphorylation by Lats kinases, YAP/TAZ are sequestered in the cytoplasm and undergo ubiquitination-mediated degradation. YAP/TAZ are also inhibited by interaction with cell junction proteins including angiomotin and α-catenin. Moreover, as transcription co-activators, YAP/TAZ need to associate with DNA-binding proteins such as TEAD family transcription factors to induce gene expression. Hence, the activity and specificity of YAP/TAZ in gene expression is also dependent on their nuclear partners.

Keywords Hippo • YAP • TAZ • Organ size • Cancer • Stem cell • Phosphorylation • Ubiquitination • Transcription

Organ size regulation is fundamental in biology and is critical not only during development but also in adulthood. A key determinant of organ size is the number of cells. Thus, organ size control is largely dependent on modulation of cell numbers.

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_5,

The Hippo signaling pathway, initially identified in *Drosophila,* has important roles in regulating cell proliferation and cell death, which consequently determines cell numbers, tissue growth, and organ size.

Hippo (Hpo), a *Drosophila* serine/threonine kinase, has been named after a massive overgrowth phenotype resulting from its genetic inactivation (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Wu et al. 2003). Additional core components of the Hippo pathway, such as Sav, Wts, and Mats, were defined similarly by genetic screens in *Drosophila* (Justice et al. 1995; Kango-Singh et al. 2002; Lai et al. 2005; Tapon et al. 2002; Xu et al. 1995). The transcription co-activator Yki mediates the biological functions of the Hippo pathway by regulating a broad transcription program (Goulev et al. 2008; Huang et al. 2005; Zhang et al. 2008; Zhao et al. 2008).

Organ size regulation by the Hippo pathway is evolutionarily conserved in mammals. Furthermore, dysregulation of this pathway leads to hyperplasia and tumorigenesis (reviewed by Zhao et al. (2010a)). The mammalian Hippo pathway is composed of a kinase cascade consisting of mammalian STE20-like protein kinase 1/2 (MST1/2, Hpo ortholog) and large tumor suppressor homolog 1/2 (Lats1/2). MST1/2, in complex with its regulatory protein Salvador (Sav), phosphorylates and activates Lats1/2 kinases (Callus et al. 2006; Chan et al. 2005). Lats1/2 also forms a complex with regulatory protein Mobkl1A/Mobkl1B (Mats ortholog, collectively referred to as Mob1 below) and phosphorylates the transcription co-activators Yes-associated protein (YAP, Yki ortholog) and WW domain containing transcription regulator 1 (WWTR1, also known as TAZ, a YAP paralog) (Chow et al. 2010; Hao et al. 2008; Lei et al. 2008; Zhao et al. 2007). YAP/TAZ are two major downstream effectors mediating functions of the mammalian Hippo pathway in development, organ size control, and tumorigenesis.

5.1 Biological Functions of YAP/TAZ

Functions of YAP and TAZ overlap but are not completely redundant, as revealed by the different phenotypes in YAP or TAZ knockout mice. YAP-null mice die at embryonic day 8.5 (E8.5), with defects in yolk sac vasculogenesis, chorioallantoic fusion, and body axis elongation (Morin-Kensicki et al. 2006), suggesting that YAP plays an important function in development. In contrast, TAZ knockout mice are viable but predisposed to renal and pulmonary diseases (Hossain et al. 2007; Makita et al. 2008; Tian et al. 2007). Furthermore, YAP/TAZ double knockout mice die before the morula stage (16–32 cells), prior to embryo implantation, indicating essential roles of YAP/TAZ in early embryonic development (Nishioka et al. 2009).

YAP/TAZ also play important roles in stem cell self-renewal and differentiation (Liu et al. 2012a). YAP activity declines when stem cells undergo differentiation (Lian et al. 2010; Tamm et al. 2011), and YAP and TAZ are required for maintaining the pluripotency of mouse and human stem cells, respectively (Alarcon et al. 2009;

Lian et al. 2010; Varelas et al. 2010b). In addition, overexpression of YAP or knockdown of Lats2 increases the induction efficiency of induced pluripotent stem (iPS) cells (Lian et al. 2010; Qin et al. 2012). In transgenic animals, enhanced YAP activity expands tissue-specific stem cells in liver, intestine, skin, and neural tube (Benhamouche et al. 2010; Camargo et al. 2007; Cao et al. 2008; Lee et al. 2010; Lu et al. 2010; Schlegelmilch et al. 2011; Song et al. 2010; Zhang et al. 2011; Zhou et al. 2011). These observations collectively demonstrate an important function of YAP/TAZ in both embryonic and tissue-specific stem cells.

Given the importance of the Hippo pathway in cell number control, it is no surprise that alteration of this pathway contributes to tumor development. Indeed, the YAP gene locus is amplified in hepatocellular carcinoma and mammary tumors (Overholtzer et al. 2006; Zender et al. 2006), and elevated YAP or TAZ expression and nuclear localization have been frequently observed in human cancers (Chan et al. 2008; Dong et al. 2007; Steinhardt et al. 2008; Zender et al. 2006; Zhao et al. 2007). On the other hand, MST1/2 and Lats1/2 are downregulated in different type of cancers (reviewed by Zhao et al. (2010a)). In YAP transgenic mice, hyperplasia and tumors are frequently observed (Camargo et al. 2007; Dong et al. 2007). Similarly, inactivation of Hippo pathway components leads to tumor development (Lee et al. 2008, 2010; Lu et al. 2010; Song et al. 2010; Zhou et al. 2009). Moreover, neurofibromin 2 (NF2), which acts upstream of MST1/2, is a well-known human tumor suppressor (Rouleau et al. 1993; Ruttledge et al. 1994). These observations suggest that YAP/TAZ and the Hippo pathway play critical roles in cancer development.

YAP contains multiple domains, such as a proline-rich domain, TEAD-binding domain, two WW domains (or one in a shorter splicing variant), an SH3-binding motif, a transcription activation domain, a coiled-coil domain, and a PDZ-binding motif. TAZ comprises similar domains, although it lacks the proline-rich domain, the second WW domain, and the SH3-binding motif. The *Drosophila* Yki is more divergent as it lacks the proline-rich domain, SH3-binding motif, coiled-coil domain, and PDZ-binding motif (Fig. 5.1). These domains set up a platform for YAP/TAZ to form an extensive interactions with their upstream regulators and downstream effectors (reviewed by Mauviel et al. (2012)).

The activity of YAP/TAZ is tightly controlled to maintain tissue homeostasis and to prevent tumorigenesis as well as other diseases. In this chapter, we will review molecular mechanisms that regulate YAP and TAZ functions.

5.2 Regulation by Phosphorylation

Phosphorylation is the most important mechanism known to regulate YAP and TAZ activity. YAP/TAZ are phosphorylated at multiple sites, as indicated by mass spectrometry and mutagenesis analysis, and YAP/TAZ phosphorylation is subjected to dynamic regulation mainly by the Hippo pathway (Zhao et al. 2010b).

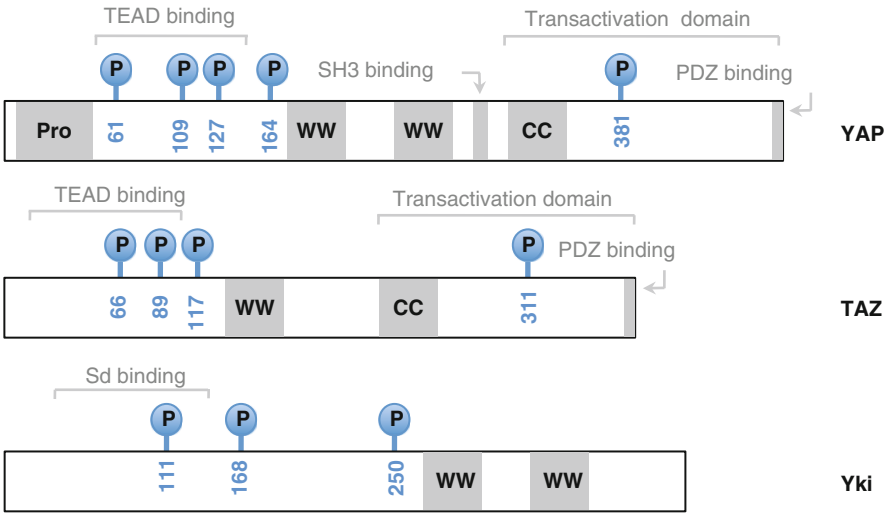

Fig. 5.1 Domain organization and Lats targeting sites on YAP/TAZ/Yki. Protein domains are illustrated using *gray* boxes. Lats1/2 phosphorylation sites (HXRXXS) on YAP, TAZ, and Yki are depicted by *blue* circles with a "P" label, and the numbers below them represent their exact positions. TEAD- or Sd-binding domains, transactivation domains, SH3-binding motif, and PDZ-binding motif are also indicated. *Pro* proline-rich domain; *WW* WW domain; *CC* coiled-coil domain

Lats1/2 have been shown to directly phosphorylate YAP at Serine 127 (S127) and TAZ at Serine 89 (S89) (Hao et al. 2008; Lei et al. 2008; Zhao et al. 2010b, 2007). Phosphorylation of YAP S127 or TAZ S89 creates a binding site for 14-3-3 (Kanai et al. 2000; Lei et al. 2008; Zhao et al. 2007). The interaction of 14-3-3 with YAP/TAZ sequesters YAP/TAZ in the cytoplasm, which results in the inactivation of YAP/TAZ transcription co-activators (Kanai et al. 2000; Lei et al. 2008; Zhao et al. 2007). In *Drosophila*, Yki is similarly repressed by wts (lats kinase ortholog) through phosphorylation and 14-3-3 binding (Oh and Irvine 2008; Ren et al. 2010). Consistently, mutation of S127 in YAP or the corresponding serine in TAZ or Yki to alanine increased YAP/TAZ/Yki nuclear localization and activity (Camargo et al. 2007; Lei et al. 2008; Oh and Irvine 2008; Zhao et al. 2007). In search of *Drosophila* mutants resistant to hpo overexpression, which reduces organ size, Yki mutations were isolated. Interestingly, these gain-of-function Yki mutations were due to the abolishment of either the wts phosphorylation motif or the 14-3-3 binding motif in Yki (Zhao et al. 2007), thus providing convincing genetic evidence for the mechanism of Yki inhibition by wts-dependent phosphorylation.

The substrate specificity of the Lats kinases is defined as an HXRXXS consensus motif (Zhao et al. 2010b). In addition to S127, YAP has four additional HXRXXS sites being phosphorylated by Lats kinases (Fig. 5.1) (Zhao et al. 2010b). Phosphorylation of the other four sites may further inactivate YAP, because a YAP

mutant with all five Lats targeting sites mutated (YAP-5SA) is more active in inducing gene expression and promoting cell growth than the S127A single mutant. The 5SA mutant is able to potently transform NIH-3T3 cells when overexpressed (Zhao et al. 2010b). Among the five Lats target sites in YAP, S127 and S381 are most critical, and a YAP with S127A and S381A double mutations is sufficient to transform NIH3T3 cells (Zhao et al. 2010b). There are additional Lats phosphorylation sites on TAZ and Yki as well (Fig. 5.1), and these sites are also important in regulating the activities of TAZ and Yki, respectively (Lei et al. 2008; Ren et al. 2010). Unlike S127 phosphorylation, which mainly exerts its inhibitory effect through 14-3-3 binding, the functions of phosphorylation at additional Lats target sites are less clear (also see below).

Inactivation of YAP or TAZ can be achieved by upregulating the activity of Lats kinases (reviewed by Zhao et al. (2010a)). In addition, phosphatases may antagonize the function of Lats kinases on YAP/TAZ by dephosphorylation. It has been shown that protein phosphatase 1 (PP1) physically interacts with TAZ (Liu et al. 2011). PP1 can promote dephosphorylation of TAZ at S89 and S311, stabilize TAZ, and induce TAZ nuclear localization, which in turn induces transcriptional activity (Liu et al. 2011). The interaction between PP1 and TAZ is strengthened by ASPP2, a known phosphatase regulatory subunit (Liu et al. 2011). Recently, another study has demonstrated a similar role of PP1 in YAP dephosphorylation (Wang et al. 2011a). In addition to PP1, PP2A has also been shown to dephosphorylate YAP in vitro (Schlegelmilch et al. 2011). Although phosphatases have been implicated in YAP/TAZ regulation, it is generally unknown how YAP/TAZ dephosphorylation is regulated and how the action of phosphatases coordinates with Lats kinases.

5.3 Regulation by Protein Stability

The protein turnover of YAP/TAZ in cells is dependent on both protein synthesis and degradation. The half-life of TAZ is about 1–2 h, while YAP is significantly more stable (Liu et al. 2010; Vigneron et al. 2010). YAP is more stable in low density cells than in high density cultures. When cells are cultured at low density, YAP/TAZ are hypophosphorylated, more stable, and tend to accumulate (Liu et al. 2010; Zhao et al. 2010b), indicating that phosphorylation may play a role in regulating the protein stability of YAP/TAZ.

Phosphorylation on YAP S381 is one of the key phosphorylation events necessary for triggering YAP degradation (Zhao et al. 2010b). Phosphorylation on S381 primes a subsequent phosphorylation on S384 and possibly S387 by another kinase, likely casein kinases 1 (CK1δ/ε, Fig. 5.2) (Zhao et al. 2010b). The amino acid sequence around S384 (DSGLS) is similar to the canonical phosphodegron DpSGXXpS recognized by β-transducin repeat-containing proteins (β-TRCP), a F-box protein which determines selectivity of SCF E3 ubiquitin ligase (Fuchs et al. 2004). Indeed, $SCF^{\beta\text{-TRCP}}$ physically interacts with YAP, and the interaction is

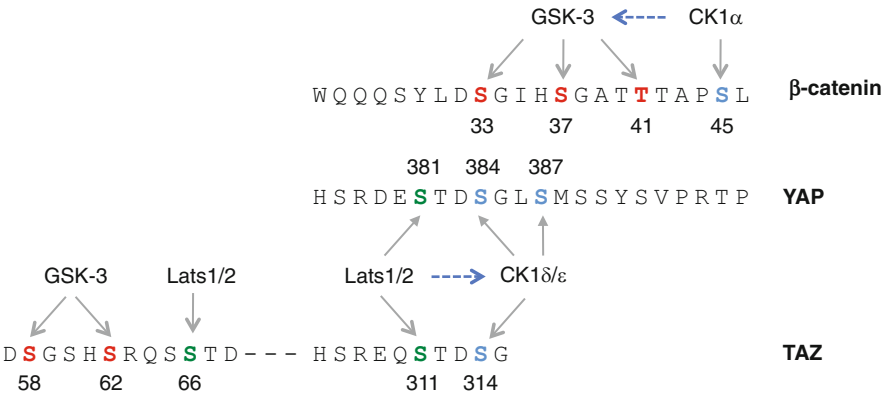

Fig. 5.2 Sequencing phosphorylation on YAP/TAZ and β-catenin. The amino acid sequences near phosphodegrons on YAP, TAZ, and β-catenin are aligned. Amino acids representing phosphorylation sites are given in *bold* face, with exact positions indicated by numbers above or below. Lats1/2, CK, and GSK-3 target sites are highlighted in *green*, *blue*, and *red*, respectively. CK1α phosphorylates S45 of β-catenin, and this phosphorylation will prime a subsequent phosphorylation on S33, S37, and T41 by GSK-3. Similarly, phosphorylation on S381 of YAP by Lats1/2 will prime phosphorylation on S384 and S387 by CK1δ/ε, and phosphorylation on S311 of TAZ by Lats1/2 will prime phosphorylation on S314 by CK1δ/ε. The priming relationships are depicted by *dished blue arrows*

facilitated by phosphorylation on YAP S381, S384, and S387 (Zhao et al. 2010b). The interaction between $SCF^{\beta\text{-TRCP}}$ and YAP induces YAP ubiquitination and eventually degradation (Zhao et al. 2010b). The phosphorylation of phosphodegron, the interaction between YAP and $SCF^{\beta\text{-TRCP}}$, and YAP degradation are all dependent on phosphorylation on S381, suggesting that the sequential posttranslational modifications on YAP are physiologically regulated by the Hippo pathway (Fig. 5.2). In addition, similar to S127A/S381A YAP double mutants, S127A/S384A and S127A/D383A double mutants can also transform NIH-3T3 cells (Zhao et al. 2010b), indicating that the phosphodegron is critical to the oncogenic activity of YAP, probably by regulating YAP protein stability.

The protein stability of TAZ is regulated in a manner similar to the regulation of YAP by Lats kinases, CK1, and $SCF^{\beta\text{-TRCP}}$ (Liu et al. 2010). Phosphorylation of TAZ S311 by Lats kinases primes subsequent phosphorylation on S314 in the phosphodegron by CK1ε and recruitment of the $SCF^{\beta\text{-TrCP}}$ E3 ubiquitin ligase, thus leading to TAZ ubiquitylation and degradation (Liu et al. 2010). TAZ has an additional phosphodegron at the N-terminal targeted by $SCF^{\beta\text{-TrCP}}$ E3 ubiquitin ligase, which may contribute to the lower protein stability of TAZ compared to YAP (Huang et al. 2012b; Liu et al. 2010; Tian et al. 2007). Phosphorylation of the C-terminal phosphodegron in TAZ is regulated by Lats, supporting TAZ stability control by the Hippo pathway. However, the N-terminal phosphodegron in TAZ is primarily controlled by GSK-3, and the latter is inhibited by PI3K and AKT pathway (Huang et al. 2012b). Activation of PI3K or PTEN mutation frequently occurs in human cancers,

which may lead to AKT activation, GSK-3 inhibition, and TAZ accumulation; upregulation of TAZ protein level may stimulate cell proliferation and contributes to cancer driven by PI3K and PTEN mutations.

The phosphodegron sequence around S381 of YAP is not conserved in *Drosophila* Yki, indicating a divergence between mammals and *Drosophila*.

5.4 Regulation by Subcellular Localization via Protein–Protein Interaction

YAP and TAZ are transcription co-activators and are required to enter the nucleus and access target transcription factors and gene promoters to exert their role in gene expression. Due to their oncogenic potential, the nuclear localization of YAP and TAZ is restricted in vivo by multiple mechanisms. As mentioned above, the phosphorylation of S127 on YAP or S89 on TAZ creates a binding site for 14-3-3. Binding with 14-3-3 leads to cytoplasmic retention of YAP/TAZ and prevents their nuclear entry (Kanai et al. 2000; Lei et al. 2008; Zhao et al. 2007). Other than 14-3-3, additional binding partners of YAP/TAZ have been recently identified, and these YAP/TAZ interacting proteins can also modulate YAP/TAZ cellular localizations.

Angiomotin (AMOT) family proteins have recently been identified as a YAP/TAZ interacting protein (Chan et al. 2011; Wang et al. 2011b; Zhao et al. 2011). The interaction is mediated by PP×Y motifs of AMOT and WW domain(s) of YAP/TAZ, and is not directly dependent on YAP/TAZ phosphorylation by Lats kinases (Zhao et al. 2011). AMOT can recruit YAP to different subcellular compartments, such as tight junctions and/or the actin cytoskeleton through physical interaction, thus reducing the translocation of YAP into the nucleus and resulting in decreased YAP activity (Chan et al. 2011; Wang et al. 2011b; Zhao et al. 2011). In addition, AMOT also potentiates YAP/TAZ phosphorylation at Lats target sites (Zhao et al. 2011). Therefore, AMOT may inhibit YAP/TAZ function through a direct binding and an indirect increase of YAP phosphorylation. A recent report shows that AMOT can bind to MST2, Lats2, and YAP, function as a scaffold protein for the core components of the Hippo pathway, and result in increased Lats2 kinase activity thus YAP phosphorylation (Paramasivam et al. 2011). Hippo pathway kinases MST1/2 and Lats1/2 and regulatory protein mob have been shown to be activated at the cell membrane (Hergovich et al. 2006; Ho et al. 2010), and AMOT family proteins may induce the clustering of YAP/TAZ and Hippo pathway kinases at tight junctions in response to cell density to regulate YAP/TAZ phosphorylation and activity (Paramasivam et al. 2011; Zhao et al. 2011).

A role for α-catenin in YAP localization has been recently suggested (Schlegelmilch et al. 2011; Silvis et al. 2011). In keratinocytes, α-catenin strongly co-immunoprecipitates with YAP (Schlegelmilch et al. 2011). However, the interaction between α-catenin and YAP is not direct, and 14-3-3 functions as a mediator for this interaction (Schlegelmilch et al. 2011). Furthermore, only phosphorylated (S127) YAP can form a complex with α-catenin because 14-3-3 recognizes

phosphorylated YAP (Schlegelmilch et al. 2011). It is known that α-catenin is a component of adherent junctions; therefore, a tripartite complex of α-catenin, 14-3-3, and YAP may sequester YAP at cell adherent junctions and prevent YAP dephosphorylation, nuclear translocation, and target gene expression. The inhibition of YAP by α-catenin may contribute to the tumor suppressor function of α-catenin (Schlegelmilch et al. 2011; Silvis et al. 2011). Phosphorylated TAZ also interacts with 14-3-3 (Kanai et al. 2000; Lei et al. 2008), but it is unclear if TAZ localization is also regulated by α-catenin. Moreover, whether the inhibitory role of α-catenin on YAP is conserved in tissues other than skin remains unknown.

Other components or regulators of cell junctions such as ZO-1, ZO-2, and PTPN14 have also been suggested as regulators of YAP/TAZ localization and activity (Huang et al. 2012a; Liu et al. 2012b; Oka et al. 2010, 2012), suggesting that sequestration of YAP/TAZ at cell junctions is a common mechanism to restrict the growth-promoting activity of YAP/TAZ.

Yki also physically interacts with upstream components of the Hippo pathway, such as Expanded, Wts, and Hpo; these interactions will restrict Yki activity by restraining Yki in the cytoplasm (Badouel et al. 2009; Oh et al. 2009). However, an ortholog of AMOT is not present in *Drosophila* and thus AMOT-dependent regulation of YAP/TAZ localization is not evolutionary conserved.

5.5 Regulation by Transcription Factor Target Selection

YAP/TAZ are both transcription co-activators without DNA-binding ability. In order to induce gene transcription, they must interact with specific transcription factors that bind to promoters of target genes. Target transcription factors selection therefore provides an additional layer of complexity to YAP/TAZ regulation.

TEAD family transcription factors (TEAD1-4) have been shown to serve as the major target transcription factors mediating the biological functions of YAP/TAZ (Vassilev et al. 2001; Zhao et al. 2008). In a functional screen of a human transcription factor library, TEADs were identified as transcriptional factors most potently activated by YAP (Zhao et al. 2008). Indeed, TEAD1/2 and YAP share a largely overlapping set of target genes (Ota and Sasaki 2008; Zhao et al. 2008). Downregulation of TEADs or disruption of the YAP–TEAD interaction blunts the expression of most YAP targeting genes and largely diminishes the ability of YAP to promote cell proliferation, cell transformation, EMT, cell contact inhibition, and maintenance of stem cell pluripotency (Lian et al. 2010; Ota and Sasaki 2008; Schlegelmilch et al. 2011; Zhao et al. 2008). In addition, TEAD1/2 double knockout mice exhibit reduced cell proliferation and enhanced apoptosis (Sawada et al. 2008), and these phenotypes are similar to those of YAP knockout mice (Zhang et al. 2010). Similarly, TEADs also interact with TAZ and mediate the function of TAZ on cell growth and EMT (Chan et al. 2009; Mahoney et al. 2005; Zhang et al. 2009). These findings indicate that TEADs serve as the major transcription factors mediating the function of YAP/TAZ in gene expression and organ size control.

YAP and TEAD form a strong physical interaction, and the detailed molecular mechanism of YAP–TEAD interaction is revealed by structural studies. Three-dimensional structures of a human YAP and TEAD1 complex (Li et al. 2010) and a mouse YAP and TEAD4 complex (Chen et al. 2010) have been resolved, although both studies used the YAP-binding domain of TEAD and the TEAD-binding domain of YAP rather than full length proteins. The complex structures indicate that the C-terminal domain of TEAD forms a globular structure with a β-sandwich fold surrounded by four α-helices on one side, with the N-terminal domain of YAP wrapping around the TEAD to form extensive interactions (Chen et al. 2010; Li et al. 2010). The crystal structure of the YAP-binding domain of human TEAD2 has also been resolved, and it adopts an immunoglobulin-like β-sandwich fold with two extra helix-turn-helix inserts (Tian et al. 2010).

The YAP–TEAD complex structures clearly show that YAP S94 directly forms a hydrogen bond with TEAD1 Y406 (Chen et al. 2010; Li et al. 2010). This provides a beautiful molecular explanation of the disrupted interaction between YAP and TEAD1 by either YAP S94 or TEAD1 Y406 mutations (Zhao et al. 2008). The TEAD1 Y406H mutation is causal to Sveinsson's chorioretinal atrophy, also referred to as helicoid peripapillary chorioretinal degeneration (Fossdal et al. 2004). Therefore, the structural and biochemical studies revealed that a disruption of YAP–TEAD1 interaction due to mutations in TEAD1 might be the underlying molecular basis for this human genetic disorder. The YAP–TEAD interaction requires only a small region of YAP; short peptides and small molecules have been shown to disrupt the interaction and reduce YAP activity in vivo (Liu-Chittenden et al. 2012; von Gise et al. 2012), and inhibitors targeting YAP/TAZ–TEAD interactions provide new therapeutic strategies to fight diseases caused by dysregulated YAP/TAZ activity, such as cancers.

Smad1, a transcription factor in the BMP signaling pathway, has been reported as YAP-interacting protein (Alarcon et al. 2009). The interaction between Smad1 and YAP is dependent on BMP signaling; following BMP stimulation, the linker region of Smad1 undergoes phosphorylation, and phosphorylated Smad1 then interacts with YAP via WW domains (Alarcon et al. 2009). The phosphorylation at the linker region of Smad1 is required for Smad1–YAP interaction, as the interaction is decreased when the phosphorylation sites are mutated (Alarcon et al. 2009). YAP has also been shown to mediate BMP target gene expression in mouse embryonic stem cells, which rely on BMP signaling for maintenance of pluripotency (Alarcon et al. 2009). In consistence, a critical role of YAP in maintaining pluripotency of mouse embryonic stem cells has been reported (Lian et al. 2010).

Smad2 and Smad3, two transcription factors in the TGFβ signaling pathway, can bind to the coiled-coil domain of TAZ (Varelas et al. 2008). TAZ also interacts with MED15, a component of the mediator complex important for gene transcription (Varelas et al. 2008). In a TGFβ signaling sensitive manner, TAZ recruits both the Smad2/3/4 complex and mediator complex to promoters of TGFβ target genes to induce transcription (Varelas et al. 2008). Knockdown of TAZ not only impairs TGFβ-induced gene expression but also promotes human stem cell differentiation (Varelas et al. 2008), suggesting that TAZ is required for the TGFβ signaling to maintain stem cell pluripotency.

YAP can also interact with Smad7, a Smad protein that inhibits both TGFβ and BMP signaling, and the interaction is mediated by the PPxY motif on Smad7 and WW domain on YAP (Ferrigno et al. 2002). Though both WW and coiled-coil domains are largely conserved in YAP and TAZ, it would be interesting to know how YAP and TAZ can interact with different Smad proteins regulated by BMP or TGFβ. A more detailed study on the relationship among YAP, TAZ, and different Smad proteins is required to address this issue. Nevertheless, these studies suggest YAP and TAZ may function in a distinct manner in human and mouse stem cells.

Besides TEADs and Smad proteins, RUNX1/2 has also been shown to interact with YAP/TAZ (Yagi et al. 1999). In addition, YAP has been shown to interact with p63, p73, and ErbB4 (Komuro et al. 2003; Omerovic et al. 2004; Strano et al. 2001). These interactions may regulate transcription of diverse genes related to cell proliferation and development.

In *Drosophila*, Sd (TEAD ortholog) genetically and physically interacts with Yki and is required for Yki-induced gene expression and tissue overgrowth (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008; Zhao et al. 2008). Although Sd is a major transcription factor mediating Yki function, the Yki mutant has a more dramatic growth defect than the Sd mutant and regulates expression of a broader range of genes than Sd (Huang et al. 2005; Wu et al. 2008), suggesting additional transcription factors acting downstream of Yki. Indeed, other transcription factors such as Mad or a complex of homothrorax (Hth) and Teashirt (Tsh) have been shown to mediate part of Yki activity in inducing microRNA *bantam* (Nolo et al. 2006; Oh and Irvine 2011; Peng et al. 2009; Thompson and Cohen 2006). It is clear that TEAD/Sd are key downstream transcription factors of YAP/TAZ and Yki. However, whether other transcription factors, such as Smad and p63, truly mediate the biological functions of YAP/TAZ requires further investigation. In addition, whether mammalian homologs of Hth and Tsh are involved in YAP/TAZ biology also waits to be tested.

5.6 Similarities Between YAP and β-Catenin Regulation

Representing the primary downstream effector of the Wnt signaling pathway, β-catenin also functions as a transcription co-activator and plays key roles in normal development and malignant transformation, and its activity is regulated at multiple layers similar to those in YAP/TAZ regulations.

Similar to YAP/TAZ, β-catenin is mainly regulated by protein phosphorylation, stability, and localization (MacDonald et al. 2009). In the absence of upstream Wnt signals, β-catenin is phosphorylated by a protein complex containing GSK-3, axin, and adenomatous polyposis coli (APC), and this phosphorylation promotes proteolytic degradation of β-catenin (MacDonald et al. 2009). Under Wnt stimulation the kinase activity of GSK-3 is inhibited causing cytoplasmic β-catenin to be hypophosphorylated, stabilized, and translocated into the nucleus. At the nucleus

β-catenin induces target gene transcription by interacting with TCF/LEF family transcription factors (MacDonald et al. 2009). The signal transduction from GSK-3 to β-catenin to TCF/LEF is highly homologous to the pathway from Lats1/2 to YAP/TAZ to TEAD.

YAP/TAZ and β-catenin also share the same E3 ubiquitin ligase $SCF^{\beta\text{-}TRCP}$ for ubiquitination and degradation (Clevers 2006). Binding between β-catenin and $SCF^{\beta\text{-}TRCP}$ depends strictly on multistep phosphorylation of the phosphodegron involving CK1α and GSK-3, in which CK1α phosphorylates S45 primes subsequent phosphorylation on S33, S37, and S41 by GSK-3 (Liu et al. 2002). Clearly, the sequential phosphorylation, ubiquitination, and protein degradation is a common strategy for regulating stability of YAP/TAZ and β-catenin.

YAP/TAZ are recruited to cell junction structures and exhibit extensive interactions with different cell junction proteins, especially at high cell densities (see above). β-catenin is well known as a structural component of adherent junctions, and is important for mediating cell adhesion and linking cadherins to the actin cytoskeleton (Gumbiner 1995). Retention at cell junctions might be a common mechanism for regulating functions of YAP/TAZ and β-catenin.

Accumulation of both YAP/TAZ and β-catenin oncoproteins has been reported in human cancers (reviewed in Clevers 2006; Zhao et al. 2010a). Upstream kinases and kinase-associated scaffolds of YAP/TAZ or β-catenin function as tumor suppressors, and downregulation or inactivation of these tumor suppressors may cause cancer via activation of YAP/TAZ or β-catenin. Indeed, downregulated MST1/2 and Lats1/2 expression, mutations of Sav1 or Mob1 (Chakraborty et al. 2007; Hisaoka et al. 2002; Jimenez-Velasco et al. 2005; Zhao et al. 2012), and mutations of APC and axin (Liu et al. 2000; Rubinfeld et al. 1996) have been reported in different types of human cancers. Therefore, there is an astonishingly high similarity between the regulation of YAP/TAZ and β-catenin. Experience from β-catenin research may help us study the regulatory mechanisms and functions of YAP/TAZ in the future.

The Hippo pathway also crosstalks with the Wnt pathway. Cytoplasmic TAZ can bind to and interfere with the phosphorylation of disheveled (Dvl), leading to β-catenin degradation (Varelas et al. 2010a). Another report shows that both YAP/TAZ can interact with β-catenin and prevent translocation of β-catenin into the nucleus (Imajo et al. 2012). In both cases, decreased Hippo signaling leads to nuclear accumulation and activation of β-catenin and YAP/TAZ. When YAP is overexpressed in mouse intestinal epithelium, total and nuclear β-catenin is increased (Camargo et al. 2007). In addition, heart-specific inactivation of Sav in mice increases heart size and at the same time enhances Wnt signaling (Heallen et al. 2011). These in vivo observations again support a positive role of YAP/TAZ on β-catenin activation.

Interesting genetic and biochemical studies within the last 10 years have revealed that the Hippo pathway plays a major role in organ size control, and that dysregulation of this pathway contributes to either tumor growth or atrophy. YAP/TAZ co-activator inhibition represents the primary outcome of the Hippo pathway, which is accomplished through a phosphorylation-dependent cytoplasmic retention and

degradation. However, not much is known about upstream regulators of the Hippo pathway. Future studies on Hippo pathway signaling cascade will lead to a better understanding of organ size control and pathobiology of tumorigenesis.

Acknowledgments We apologize for being unable to cite some primary work due to space limitations. We would like to thank Jenna Jewell and Jessica Zhou for critical reading of this manuscript. This work was supported by grants from NIH and CIRM to K.-L.G.

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Chapter 6
Regulation of YAP and TAZ by Epithelial Plasticity

Stefano Piccolo and Michelangelo Cordenonsi

Abstract The Hippo transducers YAP and TAZ are central mediators of organ growth and tumorigenesis, regulating cell proliferation, differentiation, and epithelial stemness. In this chapter, we summarize recent findings linking the activation of YAP and TAZ to the cell's structural and architectural features, such as cell polarity, cell shape, cell adhesion, and cytoskeletal dynamics. We examine how epithelial "plasticity" induced by epithelial-to-mesenchymal transition (EMT) promotes "Cancer Stem Cell" identity and YAP/TAZ activation, and discuss the role of TAZ as molecular determinant of self-renewal and tumor-seeding potentials in cancer cells. YAP and TAZ activation can also induce EMT, generating a self-sustaining loop. We then place special emphasis on biomechanical cues as regulators of epithelial plasticity, and as dominant regulators of YAP and TAZ nuclear localization and transcriptional activities. This regulation is mediated by physical forces, such as rigidity of the extracellular matrix, and tension of the actomyosin cytoskeleton. These mechanical signals hold in shape individual cells and whole tissues, and are severely disturbed in cancer. In sum, we highlight new mechanisms of YAP and TAZ regulation by cell polarity and mechanical cues. This potentially adds a new dimension to our understanding of physiology and tumorigenesis, whereby the behavior of individual cells is dictated by the integration of information about tissue architecture and mechanics mediated by YAP and TAZ.

Keywords Epithelial plasticity • Epithelial-to-mesenchymal transition • Cancer stem cells • Apico-basal polarity • Biomechanical signals • Mechanotransduction • Tissue architecture • Scribble • Hippo • Yap and TAZ

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_6,

6.1 Overview

The Hippo pathway plays fundamental roles in the control of cell proliferation, cell survival, and cell fates in development and tissue homeostasis (Halder and Johnson 2011; Pan 2010; Ramos and Camargo 2012; Zhao et al. 2010). At the centerpiece of this signaling cascade are the transcriptional cofactors YAP and TAZ. In its most basic formulation, the pathway operates as follows: YAP and TAZ are inhibited by phosphorylation mediated by the LATS1/2 kinases, that, in turn, are activated by MST1/2 kinases, the homologues of *Drosophila* Hippo (Pan 2010). The mechanism of YAP/TAZ inhibition by phosphorylation is dual: degradation by the proteasome and/or sequestration in the cytoplasm by anchoring proteins (Pan 2010; Zhao et al. 2010). In recent years, however, several variations on this basic signaling module have been reported, including LATS-independent phosphorylation of YAP/TAZ, MST-independent activation of LATS, and phosphorylation-independent modalities of YAP/TAZ regulation (Dupont et al. 2011; Moleirinho et al. 2012; Schlegelmilch et al. 2011; Zhou et al. 2009). As such, the reader should be aware of a semantic issue, as the definition of what "is" the Hippo pathway has progressively blurred to include clear non-Hippo regulations and probably other pathways feeding on YAP/TAZ activity.

A main question in the Hippo field is how the activity of YAP and TAZ is regulated by extrinsic and intrinsic cellular signals. In this chapter we will summarize some emerging paradigms of YAP and TAZ regulation by epithelial-to-mesenchymal transition (EMT) and by changes in cell shape triggered by mechanical signals that the cell receives from its microenvironment. We start with the EMT phenomenon, a profound change in cell morphology that occurs during development, tissue regeneration, and tumor progression (see Sect. 1). Several lines of evidence link EMT to acquisition of phenotypic traits typical of stem cells, in both physiological and neoplastic contexts (outlined in Sect. 2) (Polyak and Weinberg 2009). EMT promotes loss of cell polarity and loss of cell–cell adhesion (Thiery et al. 2009), two events that have been long implicated in the regulation of the Hippo pathway in *Drosophila* and mammalian cells (Genevet and Tapon 2011).

YAP and TAZ have been also shown to be critical in controlling the amplification of stem cells and tissue progenitor cells in several tissues, playing important roles in tissue regeneration (Ramos and Camargo 2012). These functions are diverted in cancer, where YAP and TAZ serve as potent promoters of malignancy and of cancer cell "stemness," thus recapitulating the effects of EMT (Bhat et al. 2011; Camargo et al. 2007; Cao et al. 2008; Cordenonsi et al. 2011; Lee et al. 2010; Lu et al. 2010; Moleirinho et al. 2012; Pan 2010; Schlegelmilch et al. 2011; Song et al. 2010; Zhou et al. 2011a). Despite the clear analogies between EMT and YAP/TAZ biology, only recently a direct biochemical link between EMT, cell polarity, and Hippo signaling has been revealed, along with the demonstration of a causal relationship between EMT, activation of TAZ, and induction of cancer stem cell (CSCs) traits (Cordenonsi et al. 2011) (see Sect. 3).

One of the most fascinating and recently emerged aspects of YAP and TAZ biology is their regulation by structural elements that originate at the tissue level, such

as adhesion of a cell to its surrounding extracellular matrix (ECM), cell–cell junctions and the tensional forces of the cytoskeleton that keep cells, tissues, and organs in a certain shape (Halder et al. 2012). As such, these regulations can inform individual cells about properties of the tissue in which they are embedded, such as organ size and three-dimensional organization. In other words, YAP and TAZ regulations offer the unprecedented opportunity to explore one of the Holy Grails in biological research, that is, wiring signal transduction and cell biology to the overarching tissue biology (see Sect. 4).

6.2 What is EMT?

EMT is a phenotypic switch by which epithelial cells lose cell–cell adhesion and apico-basal polarity, and instead acquire motility, invasiveness, and resistance to apoptosis (Fig. 6.1) (Thiery et al. 2009). In addition, EMT includes acquisition of spread cell morphology, extended cell-ECM contacts, and capacity to degrade the basement membrane and infiltrate the underlying stroma (Fig. 6.1). Such epithelial plasticity is critical for building organs during embryogenesis and for rapid mobilization of cells for tissue repair during adult life. However, EMT is also involved in various human pathologies, most notably fibrosis and cancer (Thiery et al. 2009).

The most remarkable feature of EMT in the context of tumor biology is endowing cancer cells with characteristics typical of "cancer stem cells", including the capacity to self-renew and to generate secondary tumors (Chaffer and Weinberg 2011; Mani et al. 2008; Polyak and Weinberg 2009). In addition, non-transformed epithelial cells may also undergo EMT and, in so doing, gain stemness to the extent that a single EMT-transited mammary cell is sufficient to generate an entire mammary gland when implanted into the mouse fat pad (Guo et al. 2012; Mani et al. 2008).

It is important to reflect on the fact EMT—and its reverse process mesenchymal-to-epithelial transition (MET)—also entail profound *morphological* changes, thus reminding us how much information can be stored in cell shape itself, irrespectively of the specific set of mutations that characterize a given cancer (Bissell and Hines 2011; Butcher et al. 2009). This provides a departure from a pure "DNA-centric" perspective in tumor biology, one whereby a specific cellular status (e.g., differentiation, capacity of tumor initiation, and chemoresistance) could be intrinsic to the shape assumed by cancer cells (i.e., epithelial vs. mesenchymal shapes). In fact, acquisition of driving mutations in potent oncogenes may not be sufficient to generate a neoplasia until tumor cells are kept in check by the three-dimensional architecture of the surrounding tissue (Bissell and Hines 2011; Podsypanina et al. 2008; Leung and Brugge 2012; Dolberg and Bissell 1984; Holst et al. 2003; Illmensee and Mintz 1976; Jonason et al. 1996; Levental et al. 2009; Michaloglou et al. 2005; Weaver et al. 1997). As such, sporadic tumor cells may remain dormant even for years before overcoming this overarching barrier, possibly by undergoing EMT. In the following section we will focus on the nature of epithelial traits targeted by the EMT, and on the intracellular and extracellular determinants of EMT.

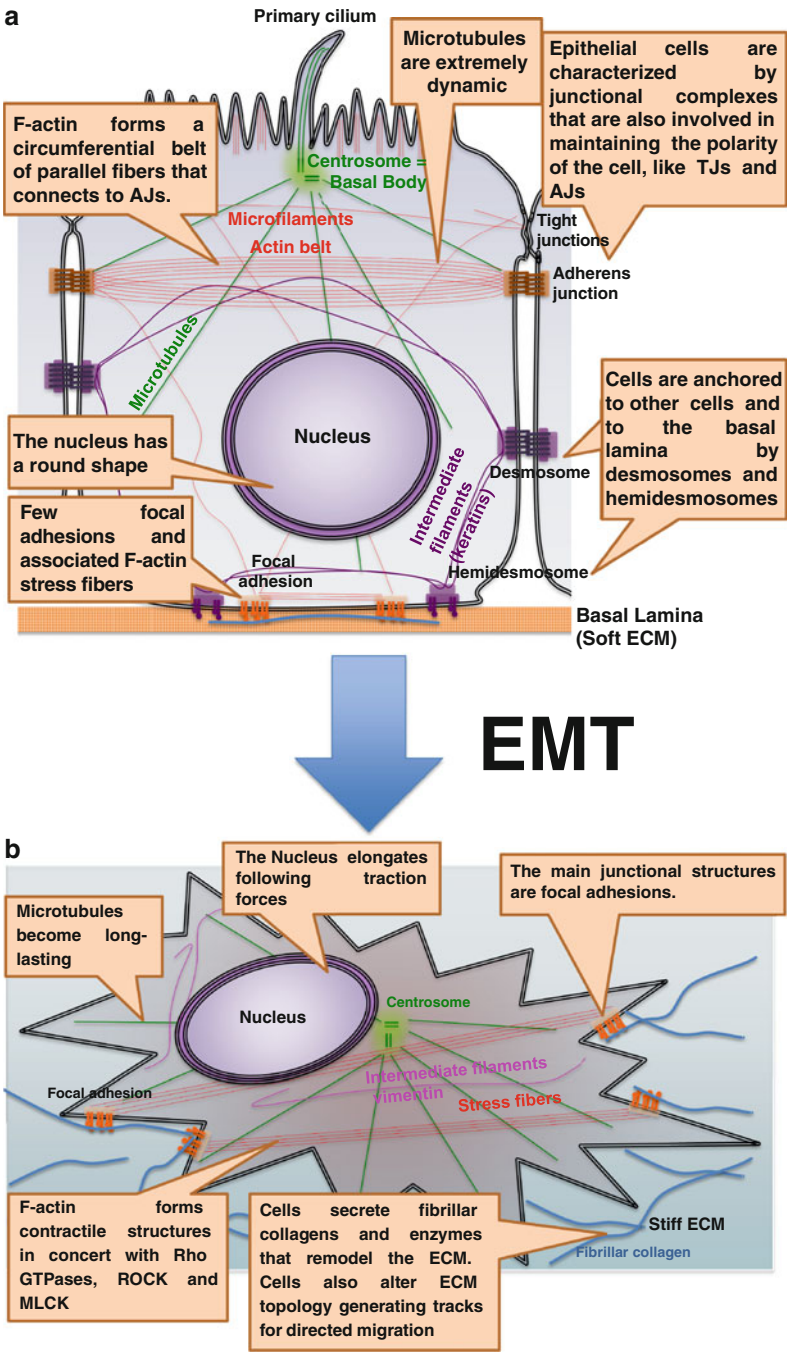

Fig. 6.1 Changes in cell architecture during EMT. See text and callouts

6.2.1 The Epithelial Format: Junctional Complexes and Apico-Basal Polarity

Most of our organs contain epithelial sheets. The epithelial format emerged more than 600 million years ago to separate the inside from the outside of multicellular organisms. Epithelia have evolved specialized structures that sustain barrier, secretion and absorption functions, but also more sophisticated activities including maintenance of tissue architecture, sensing cell density, and tumor suppression (Nelson and Fuchs 2010).

The sites of cell–cell and cell-substrate contacts are the "sensorial" interfaces by which cells experience their outside world. As such, these privileged areas of the cell's border are essential in many ways, including (a) to mediate "social cues," such as contact inhibition of growth; (b) to orient intracellular structures coherently within the polarity and shape of the neighboring cells; and (c) to organize the cytoskeleton, adapting it to the biomechanical properties of the rest of the tissue (Fig. 6.1a) (Halder et al. 2012).

In epithelia, cells are adjoined to each other by specific junctional complexes, namely tight junctions, adherens junctions, and desmosomes (Box 6.1), and to the ECM through focal adhesions and hemidesmosomes (Nelson and Fuchs 2010). The maintenance of cellular junctions is dependent on cell polarization along the cell's apico-basal axis; vice versa, the distinct protein complexes regulating apico-basal cell polarity (Box 6.1 and Fig. 6.2) require cell–cell adhesiveness to maintain their asymmetric localization (Martin-Belmonte and Perez-Moreno 2011). This mutually reinforcing adhesion-polarity loop provides a tumor suppressive environment to epithelial tissues. The EMT targets this system at the heart (Moreno-Bueno et al. 2008). Indeed, EMT invariably entails the loss of E-cadherin (or its cytoplasmic relocalization) causing loss of cell–cell adhesive capacity. This leads to cell depolarization that, in a vicious loop, causes further dismantling of cell–cell adhesion (Martin-Belmonte and Perez-Moreno 2011; Thiery et al. 2009). In turn, this reflects into an increased dependency on cell-ECM adhesion that licenses cell proliferation or survival (Livshits et al. 2012; Nelson et al. 2004; Paszek et al. 2005; Ruiz and Chen 2008). Furthermore, since junctional complexes have evolved tight connection to several key signal transduction pathways (Nelson and Fuchs 2010)—and to the Hippo pathway in particular (Box 6.2)—EMT entails broad effects on cell signaling.

6.2.2 Installing EMT

How is the EMT program installed? EMT is under the control of distinct environmental cues typically acting in concert with each other and whose specific relevance may depend on the cellular context. This includes signaling from hypoxia and growth factors, including TGFβ, Wnt, Notch, Hedgehog, Interleukins, and RTK ligands (Polyak and Weinberg 2009; Thiery et al. 2009). The source of these signals is typically found in the stromal cells, but they can also be produced by epithelial

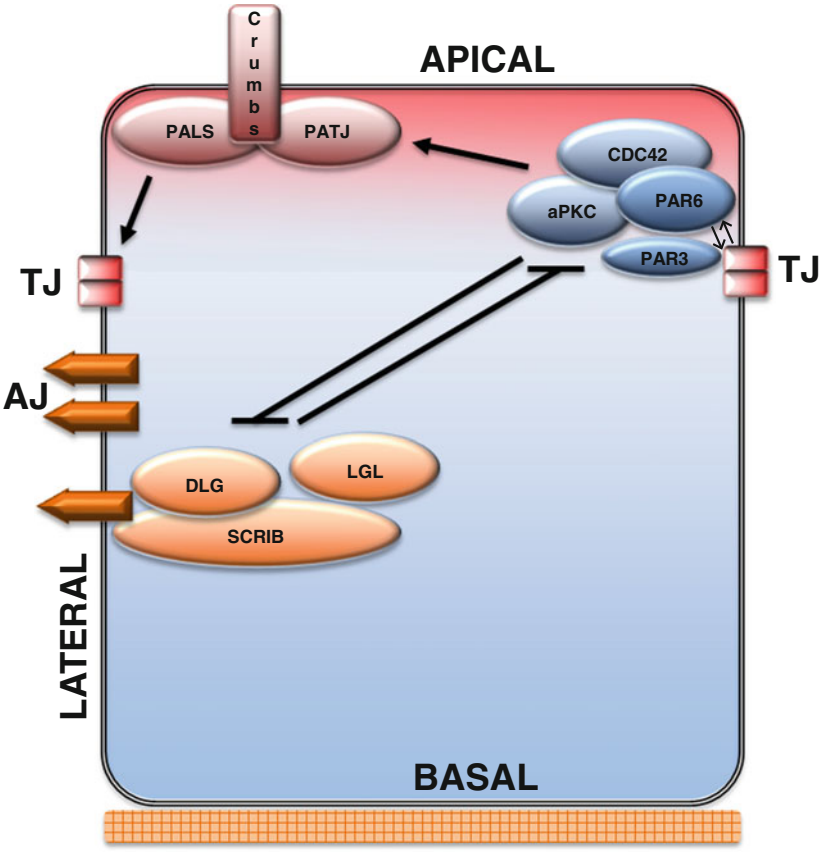

Fig. 6.2 Protein complexes regulating epithelial cell apico-basal polarity

Box 6.1 Molecular Composition of Junctional and Polarity Complexes

Several regulators of the Hippo pathway, and a number of YAP and TAZ binding proteins are involved in the establishment or maintenance of cell polarity and cell adhesion through adherens junctions and tight junctions, whose key features are described below. Cell-EMC adhesion will be discussed in the context of YAP/TAZ regulation by mechanical cues (see Sect. 4).

Tight junctions. All adhesion sites are composed by a basic module: transmembrane proteins recruit a number of cytoplasmic effectors, in turn linking the whole complex to cytoskeletal proteins. In the case of tight junctions (TJs) the main transmembrane components are claudins,

(continued)

Box 6.1 (continued)

that polymerize with each other and between adjoining cells. Other transmembrane proteins are then incorporated in such claudin mesh, including occudin and Ig-like proteins. The cytoplasmic tail of claudins interacts with ZO-1, ZO-2, ZO-3, and cingulin, that associate with F-actin (Fig. 6.1) (Nelson and Fuchs 2010).

Adherens junctions. These structures play a prominent role in connecting and transmitting forces between neighboring cells. Disruption of AJs causes loosening of cell–cell contact and disorganized tissue structure. AJs are composed of transmembrane cadherins (such as classical cadherins, e.g., E-cadherin and N-cadherin) mediating a homophilic cell–cell association, and of juxtamembrane catenins (β-, p120-, and γ-catenins) and α-catenin (Fig. 6.1). β-catenin mediates the recruitment of α-catenin, that is critical to link AJs to the actin cytoskeleton. Microtubules also associate to AJs through p120 catenin (Harris and Tepass 2010; Nelson and Fuchs 2010). The formation of TJs and of E-cadherin containing AJs is severely disturbed by EMT.

Desmosomes. These intercellular junctions provide a link between the intermediate filaments and the plasma membranes of adjoining cells, and are crucial for epithelial integrity. Intercellular adhesion is mediated by Desmoglins and Desmocollins, whose cytoplasmic domain binds to plakoglobin and plakophilins that are connected to intermediate filaments through desmoplakin. Intermediate filaments provide mechanical stability and confer resistance to mechanical stresses, due to their material properties and wiring with microtubules and microfilaments. During EMT and tumor progression, transformed epithelial cells drastically change their expression pattern of intermediate filaments, facilitating epithelial plasticity, and cell migration (Herrmann et al. 2009; Nelson and Fuchs 2010).

Apico-basal polarity complexes. There are three main cell polarity complexes in epithelial cells (see Fig. 6.2 below): the apical crumbs complex (CRB) contains the transmembrane protein CRB and the associated cytoplasmic proteins PALS1 and PATJ, and regulates the apical positioning of the TJs. The SCRIB complex—including Scribble (Scrib), Lethal Giant Larvae (LGL), and Discs Large (DLG)—organizes the basolateral plasma membrane domain, and is closely associated to the formation of AJs. The Par complex (composed of a-PCK, Par6, cdc42, and Par3) controls the activity and location of the CRB and SCRIB complexes promoting the formation of the border between apical and lateral domain. In addition, the Par and SCRIB complexes reciprocally inhibit each other, contributing to the robust spatial separation between different apical- and basal-domains (Martin-Belmonte and Perez-Moreno 2011).

Box 6.2 Cell–Cell Adhesion and Cell Polarity Complexes as Regulators of the Hippo Pathway

One of the long-standing issues in the Hippo field relates to the biochemical mechanisms by which this pathway is regulated upstream of the Hippo kinases MST1/2 and LATS1/2. Several findings point to cell–cell adhesion and cell polarity proteins as upstream regulators of YAP and TAZ.

Role of Scribble as adaptor for YAP/TAZ regulation by the Hippo kinases. See main text.

Role of Crumbs/AMOT complex in YAP/TAZ regulation. In addition to the Scribble-Hippo-TAZ connection, there are other mechanisms by which polarity and cell–cell junctions can control TAZ and YAP activity. AMOT (Angiomotin) and ZO2 are tight-junction associated proteins identified by several groups as YAP/TAZ interacting partners (Chan et al. 2011; Oka et al. 2010; Varelas et al. 2010; Wang et al. 2010; Zhao et al. 2011). In addition to AMOT, other apical proteins, including the Crumbs-associated PALS and PATJ, associate to TAZ/YAP (Varelas et al. 2010). These associations are instrumental for attenuation of YAP and TAZ activity, but the underlying mechanism is unclear. One possibility is that these proteins may simply sequester YAP and TAZ on the plasma membrane. Another possibility is that, similarly to Scribble, these proteins may serve as a supramolecular scaffold for YAP and TAZ phosphorylation. Besides binding YAP or TAZ, PALS, PATJ, and AMOT play relevant functions for junctional integrity, cell polarization, and cytoskeletal organization. Thus, a third possibility is that the YAP and TAZ regulation by these apical proteins may be secondary to loss-of-polarity and disturbed Scribble localization, or secondary to aberrant cytoskeletal organization. More studies are required to discriminate between these possibilities.

Role of E-cadherin and α-catenin as YAP regulators. The AJ components E-cadherin and α-catenin have been also implicated in YAP regulation. Expression of E-cadherin restores the density-dependent nuclear exclusion of YAP in mesenchymal cells; conversely, disturbing the E-cadherin/α-catenin complex in epithelial cells decreases phosphorylation of YAP and promotes YAP nuclear accumulation (Kim et al. 2011).

In a different study, epidermal-specific genetic depletion of α-catenin also leads to YAP dephosphorylation and nuclear accumulation (Schlegelmilch et al. 2011). The phenotype of these α-catenin mutants recapitulates the effect of YAP overexpression in transgenic mice. Interestingly, however, neither MST1/2 nor LATS1/2 are implicated in the regulation of YAP by α-catenin in keratinocytes, suggesting that other kinases may act redundantly with the canonical Hippo kinases in this and perhaps other cellular contexts.

cells themselves in an autocrine manner (Scheel et al. 2011). In addition, ECM stiffness is also a potent inducer of EMT-like effects (see Sect. 4 below) (Gjorevski et al. 2012). It is important to note that most of these cues have been implicated in directing differentiation or proliferation in embryonic development, or as "niche" factors for adult stem cells (Dreesen and Brivanlou 2007). This suggests that the EMT program in cancer cells may represent a hijacking of normal mechanisms of tissue formation and maintenance.

Despite the diversity of these inputs, the EMT program follows a rather stereotyped set of events. Indeed, directly or indirectly, all these inputs converge on the regulation of a group of transcription factors able to repress epithelial gene expression. This group includes Snail (SNAI1), Slug (SNAI2), ZEB1/2, and Twist (Peinado et al. 2007). Overexpression of individual members of this group can orchestrate the EMT program, instill stemness and activate the invasion-metastasis cascade (Guo et al. 2012; Wellner et al. 2009). For example, members of the TGFβ family are among the most extensively studied inducers of EMT (Heldin et al. 2009; Zavadil and Bottinger 2005). TGFβ induces EMT by inducing expression of ZEB and Snail proteins that turn off the epithelial program by repressing expression of E-Cadherin and of other junctional and polarity proteins. TGFβ can also foster EMT indirectly through activation of Rho GTPases (Bhowmick et al. 2001; Peinado et al. 2007; Wang et al. 2006; Zavadil and Bottinger 2005).

One of the mysteries of the EMT process is its duration and stability: EMT is mostly a transient phenomenon in vivo raising questions on what regulates the rate of conversion between the epithelial and EMT states (Chaffer and Weinberg 2011; Thiery et al. 2009). Work in the microRNA field has shown that mutual repression between a miRNA and its target is a very effective way to generate all-or-none responses and "one-of-the-two" cellular decisions (Inui et al. 2010). The reciprocal antagonism between miR-200 and ZEB1 or ZEB2 represents a paradigm for this feedback module. miR-200 family members are expressed in epithelial cells, where they inhibit ZEB1/2 expression preserving E-Cadherin expression; however, in mesenchymal cells, ZEB1 and ZEB2 transcriptionally repress miR-200 transcription (Gregory et al. 2008; Inui et al. 2010; Wellner et al. 2009). This mutual inhibitory loop provides robustness to the EMT phenomena, as EMT-inducing stimuli must be present for sufficient time and intensity at least to surpass the miR-200 barrier.

6.3 EMT and the Cancer Stem Cells Phenomenon

6.3.1 Cancer Stem Cells

Tumor cells are phenotypically heterogeneous, raising the question as to how this diversity is generated. In the classical view, intratumoral heterogeneity is caused by the tumor's intrinsic genetic instability, spawning many genetically distinct subclones,

sorted by Darwinian selection (Shackleton et al. 2009). Moreover, in recent years, it has become increasingly clear that a tumor is not a randomly organized collection of cells; rather, a tumor should be better envisioned as an aberrant attempt at de novo organogenesis, or as an organ "caricature" still taking advantage of the same molecular and cellular mechanisms utilized during development for epithelial self-renewal and differentiation (Egeblad et al. 2010; Pierce and Speers 1988). In fact, recapitulating the cellular hierarchies of normal tissues, tumors include a specific cell subpopulation of cancer cells—termed cancer stem cells (or tumor-initiating cells)—lodged into specific environmental niches and responsible for constant tumor regeneration (Nguyen et al. 2012; Shackleton et al. 2009; Visvader and Lindeman 2012). CSCs are operationally defined as the fraction of tumor cells specifically endowed with self-renewal, tumor-seeding, and chemoresistance potential as well as ability to generate non-CSC progeny that constitutes the rest of the tumor bulk (Visvader and Lindeman 2012).

The molecular and cellular bases of the CSC properties remain enigmatic. In fact, CSCs can be identified only retrospectively, depending on assays that measure the self-renewing potential of individual cells growing as spheres in vitro, their capacity of initiate new tumors when injected in recipient mice at limiting dilutions, or the capacity to form broad clonal descendants in vivo (Chen et al. 2012; Driessens et al. 2012; Gilbertson and Graham 2012; Gupta et al. 2009a; Nguyen et al. 2012; Schepers et al. 2012). The lack of molecular definition has contributed to a number of debates over the CSC concept, including how abundant they are, what is their relationships with normal stem cells, or whether CSCs can be generated from non-CSCs (Magee et al. 2012).

6.3.2 EMT and CSCs

What are the main evidences connecting EMT and CSCs? First, transformed human mammary epithelial cells that have undergone an EMT display an increased capacity to self-renew, to grow as soft agar colonies, and to generate tumors (Mani et al. 2008). Moreover, experimental induction of EMT promotes the formation of cells expressing cell-surface antigens that are found enriched in naturally emerging CSC populations (Mani et al. 2008). Second, EMT has been implicated in conferring metastatic potential and therapeutic resistance (Chaffer and Weinberg 2011; Gupta et al. 2009b; Moody et al. 2005; Sayan et al. 2009; Witta et al. 2006; Yang et al. 2004). The formation of secondary tumors or tumor regeneration after chemotherapy must rely on CSC-like properties. Further highlighting the link between EMT and CSCs is that fact that transcriptional inducers of EMT, such as Slug, are also instrumental for conferring stemness to normal mammary epithelia and full metastatic potential to CSC-like breast cancer cell lines (Guo et al. 2012). In line, elevated expression of EMT-inducing factors is clinically relevant, it has been detected at the tumor-stroma borders and associated to elevated incidence of metastasis,

recurrence, and poor differentiation in multiple types of tumors (Peinado et al. 2007; Polyak and Weinberg 2009).

CSC representation is strongly dependent on tumor grade (with advanced tumors containing more CSCs than differentiated tumors) and microenvironmental factors (Driessens et al. 2012; Egeblad et al. 2010; Pece et al. 2010; Visvader and Lindeman 2012). Indeed, there is substantial evidence that contextual signals may expand or shrink the pool of CSCs by tuning self-renewal, differentiation or by inducing CSC-like traits in non-CSC (Chaffer et al. 2011; Quintana et al. 2010; Roesch et al. 2010). For example, TGFb and Wnt signaling have been identified as autocrine factors that maintain the stem cell state in mammary cells and collaborate to induce EMT (Scheel et al. 2011). The dynamic equilibrium between the CSCs and non-CSCs population is reminiscent of the instability and reversibility of the EMT phenotype and is indeed regulated by the same factors that control EMT.

6.3.3 Parsing EMT

The identification of EMT as a process able to endow "stemness" to epithelial cells clearly represented a critical discovery, because it anchored the operational definitions of stem cells and CSC to well-defined and well recognizable morphological features of the cell (Mani et al. 2008). That said, most of what we know about the connection between EMT and CSCs comes from studies conducted in cells cultured in vitro or isolated ex-vivo; in contrast, transition to mesenchymal cell fates has been reported only in specific tumor subsets and appears as an overall rare event (Chaffer and Weinberg 2011; Savagner 2010). This raises questions on the general relevance of EMT in human tumors. Clearly, EMT and CSCs are broad and only recently added dimensions to the cancer field, and much research is needed to attain more definitive answers. For example, EMT may not be a general feature of the whole tumor but instead it may occur only at specific locations, such as in proximity of the activated stroma at the tumor border. Moreover, epithelial cells that acquired a full EMT might be indistinguishable from fibroblasts, posing a technical challenge to their identification by routine histopathological examination. These caveats notwithstanding, it is worth noting here that part of the problem may be in our definition of EMT. Although the term EMT was originally limited to the acquisition of a fibroblast-like, spindle cell morphology, the reality of tumors may be much more variegated. "Partial-EMTs" have been described in tumors, a condition whereby cells co-express epithelial and mesenchymal markers (Klymkowsky and Savagner 2009). More critically, not all the segments of the complex EMT program may be equally necessary to confer stemness potential. Loss of apicobasal polarity—a true hallmark of cancer, and primary initial step of any EMT—is more likely to be at the center stage of EMT-mediated induction of CSC.

6.4 EMT and HIPPO: EMT as Upstream Regulator of YAP and TAZ

In the above discussion, we have outlined some key aspects of EMT, its upstream inducers and the link to CSCs, but left unaddressed perhaps the most critical question: what are the molecular effectors downstream of EMT? What is executing the genetic programs of stemness, tumor progression and CSC-traits that are associated to EMT? Recent studies have highlighted, on the one hand, the fundamental role of YAP and TAZ as mediators of stemness in normal stem cells and cancer stem cells, and, on the other hand, the regulation of YAP and TAZ by cell polarity and EMT (Bhat et al. 2011; Cordenonsi et al. 2011). This indicated YAP and TAZ as ideal candidate to mediate some of the key biological effects of EMT.

6.4.1 The TAZ-CSCs Connection

TAZ has recently emerged as a primary molecular determinant of several characteristics of CSCs. TAZ is required for self-renewal and tumor-initiation capacities of breast cancer cells, as measured by the capacity of cells to grow as self-regenerating mammospheres and to form tumors once cancer cells are injected as limiting dilutions in immunocompromised mice (Cordenonsi et al. 2011). Notably, loss of TAZ impairs invasiveness, self-renewal, and tumorigenic capacity also in primary glioblastoma stem cells (GSCs), indicating that TAZ may also confer CSC-traits in tumors other than breast cancer (Bhat et al. 2011). Moreover, gain-of-TAZ endows these properties to otherwise non-CSC breast cancer cell populations (Cordenonsi et al. 2011). Importantly, if TAZ levels are experimentally induced in differentiated tumor cells, these cells generate high-grade/undifferentiated tumors (Bhat et al. 2011; Cordenonsi et al. 2011). Finally, TAZ expression is associated with expression of cell-surface antigens typical of putative CSC populations (Bhat et al. 2011; Cordenonsi et al. 2011).

There is substantial evidence that the proportion of CSCs is higher in poorly differentiated human primary tumors, namely, those routinely classified as “high-grade” malignancies by histopathological examination (Pece et al. 2010). TAZ protein levels are indeed elevated in high-grade breast cancers and glioblastomas, as assayed by immunohistochemistry in primary tumor samples (Bhat et al. 2011; Cordenonsi et al. 2011). The notion that TAZ is a CSC determinant is further supported by bioinformatic analyses of public datasets containing gene expression profiles and associated clinical history for a large collection of primary mammary tumors. First, signatures of TAZ activation (as defined by sets of TAZ target genes) identify the same tumors displaying signatures of “stemness genes” (Cordenonsi et al. 2011). Second, high TAZ is also an important clinical variable that discriminate tumors associated to poor survival and metastasis, and that are resistant to chemotherapy (Cordenonsi et al. 2011; Lai et al. 2011).

6.4.2 An EMT-Scribble-TAZ Axis in Breast Cancer

TAZ protein stabilization is induced by EMT-inducing transcription factors, such as Twist or Snail, and, crucially, TAZ is required for self-renewal induced by these EMT-promoting factors in breast cancer cells (Cordenonsi et al. 2011). Intriguingly, loss of TAZ does not revert mesenchymal cells back to an epithelial state; in other words, TAZ is downstream of EMT, and uncouples mesenchymality from EMT-induced stemness, being TAZ dispensable for the first and required for the second.

Recent evidence connects TAZ regulation by EMT to the basolateral polarity determinant Scribble (Box 6.1). The SCRIB complex plays a prominent role in human malignancies: it is downregulated or cytoplasmically mislocalized in a broad variety of tumors (including colon, breast, cervical, prostate, and lung) (Martin-Belmonte and Perez-Moreno 2011; Pearson et al. 2011; Vaira et al. 2011; Zhan et al. 2008). During the initial steps of EMT Scribble is delocalized from the membrane to the cytoplasm (Cordenonsi et al. 2011), likely as a consequence of cadherin downregulation (Navarro et al. 2005). Notably, Scribble inactivation—or its transient removal from the plasma membranes—is sufficient, *per se,* to increase stemness potential in mammary epithelial cells (Cordenonsi et al. 2011).

TAZ stabilization triggered by EMT is recapitulated by the sole loss-of-Scribble, indicating that loss of this polarity determinant may be sufficient to endow some of the key attributes of EMT without the need of reaching the full mesenchymal fate. Indeed, tethering Scribble to the plasma membrane can downregulate TAZ levels in cells that passed EMT (Cordenonsi et al. 2011). Mechanistically, Scribble serves as membrane-localized adaptor for TAZ and the Hippo kinases LATS and MST, leading to TAZ phosphorylation and subsequent recognition by the β-TrCP E3 ubiquitin ligase complex that causes TAZ degradation (Cordenonsi et al. 2011). Loss-of-Scribble, or its delocalization from the plasma membrane, prevents TAZ phosphorylation, leading to TAZ stabilization and nuclear activity. In addition to the Scribble-Hippo-TAZ connection, there are other mechanisms by which polarity and cell–cell junctions can control TAZ and YAP activity (see Box 6.2), although the exploitation of these mechanisms during EMT remains to be tested. We conclude that TAZ protein stabilization downstream of EMT embodies some salient characteristics so far only operationally linked to CSCs, such as tumor heterogeneity, reduced differentiation, self-renewal, tumor initiation, chemoresistance, and plasticity.

6.4.3 YAP and TAZ as Upstream Inducers of EMT

The relationship between YAP/TAZ and EMT is likely to be bidirectional, whereby EMT induces YAP and TAZ that in turn sustain the EMT program, at least in some cellular contexts. This would configure an autonomous, self-sustaining loop for enduring YAP/TAZ stabilization.

The activity of YAP as EMT inducer was noted in mammary epithelial cells since the very first report of YAP oncogenic properties (Overholtzer et al. 2006). This early observation was soon followed by others, showing that overexpression of a non-phosphorylatable form of YAP or TAZ induced EMT in MCF10A cells (Chan et al. 2008; Lei et al. 2008; Zhao et al. 2008). Thus, EMT induction in MCF10A cells is a very robust bioassay to monitor the activity of overexpressed YAP/TAZ. Little is known on how YAP and TAZ activate EMT; clearly, EMT-inducing transcription factors are likely candidates as YAP/TAZ target genes, but this hypothesis has not been tested so far.

However, EMT induction by YAP or TAZ overexpression should not be considered a general event. YAP overexpression fails to promote EMT in ovarian cancer cells or in normal bronchial epithelial cells in vitro (Zhang et al. 2011; Zhou et al. 2011b),or in hepatocytes, keratinocytes, and intestinal epithelial cells of YAP-overexpressing transgenic mice (Camargo et al. 2007; Dong et al. 2007; Schlegelmilch et al. 2011). Furthermore, endogenous YAP and TAZ activation, following genetic ablation of MST1/MST2 or Salvador (WW45), is not sufficient to trigger EMT in a variety of epithelial tissues (Lee et al. 2008, 2010; Zhou et al. 2009, 2011a). Importantly in this regard, loss of YAP or TAZ—or overexpression of a dominant-negative version of TEAD—do not typically induce gain of epithelial characteristics in mesenchymal cells (Cordenonsi et al. 2011; Dupont et al. 2011; Hong et al. 2005; Ota and Sasaki 2008). For example, as mentioned above, TAZ knockdown does not affect the mesenchymal differentiation of the metastatic or Snail-expressing breast cancer cell lines (Cordenonsi et al. 2011). The role of TAZ in high-grade glioblastoma is again a different scenario, as this is a mesenchymal type of tumor for which endogenous TAZ is pivotal for the maintenance of mesenchymal and aggressive traits (Bhat et al. 2011). How this conclusion can be generalized to other kind of mesenchymal-like tumors is unknown. Collectively these evidences indicate that YAP and TAZ can induce EMT depending on their expression levels, the experimental conditions and the cellular context.

6.5 A New Perspective on YAP/TAZ Regulation: Role of Mechanical Cues

A hallmark of cancer is the loss of tissue integrity, an event occurring even before a frank neoplasia could be even identified (Huang and Ingber 1999; Husemann et al. 2008; Podsypanina et al. 2008; Rhim et al. 2012). The identification of YAP and TAZ as downstream factors of epithelial plasticity, coupled with their potent pro-tumorigenic and pro-stemness properties, has thus clear implications in cancer research, as it sets the stage to understand molecularly the causal roles of disturbed tissue integrity for malignant transformation or dedifferentiation of tumor cells.

6.5.1 Mechanical Control of the Cancer Cell Phenotype

The above presentation has sidestepped another equally relevant issue in tumorigenesis: what is causing loss of tissue integrity in the first place? There is an increasing appreciation that mechanical inputs from the aberrant tumor microenvironment profoundly influence the tumor cell phenotype, and that may perhaps have an initiating role in tumorigenesis (Butcher et al. 2009; Egeblad et al. 2010; Huang and Ingber 2005; Jaalouk and Lammerding 2009; Provenzano and Keely 2011). Mechanical signals are pervasive elements of tissue development and homeostasis, and cells are normally subjected to different forces including stretching, compression, and pressure. These signals, typically generated by local distortions of tissue architecture, occur at the nanometer level, and thus can target individual cells with exquisite specificity; at the same time, mechanical cues can reverberate at great speed to distant cells though a "wave-like" propagation mediated by the semi-flexible and pre-tensed organization of the ECM network (Janmey and Miller 2011).

Mechanical signals become aberrant in cancer: tumor growth is typically accompanied by increased compression forces from the surrounding ECM and tissues, and by increased pressure of interstitial fluids caused by the tumor's disorganized capillaries. In addition, tumors display profound changes in ECM composition and overall increase in ECM rigidity. In particular, while the normal mammary gland is soft, breast cancers are extremely stiff, due to activation of cancer-associated fibroblasts and extensive deposition of collagen (Butcher et al. 2009). Tumors also display increased expression of lysyl-oxidases (LOX), enzymes that cross-link, and thus stiffen, the collagen fibers, and tumors with the highest expression of LOX are those displaying less differentiation and poorer prognosis (Erler et al. 2006). A causal role between stiffness of the ECM and tumor progression has been recently obtained in animal models, whereby enhancing collagen cross-linking enhanced tumor progression, while targeting LOX—and thus attenuating cross-linking—reduced tumor incidence and delayed progression(Levental et al. 2009).

6.5.2 Form is Function: The Control of Cell Behavior by Cell Shape

The rigidity of the ECM is perceived by cells as an increased resisting force; this force is transmitted through integrins to the cell's cytoskeleton (Parsons et al. 2010; Vogel and Sheetz 2006). On a stiff ECM the cell increases its inner pulling forces, namely, the tension of the actomyosin cytoskeleton, in order to balance the strong external resisting forces. As such, to an increase in ECM rigidity corresponds an increased intracellular stiffness (Ingber 2006; Mammoto et al. 2009; Parsons et al. 2010; Provenzano and Keely 2011).

Mechanical inputs can be perceived by focal adhesions, or transmitted by cell–cell junctions from neighboring cells, all impacting and depending on the organization of the cytoskeleton (Mammoto et al. 2009; Parsons et al. 2010). Thus, cells are mechanically connected to their surroundings (cells, ECM, and whole tissue) in a manner that is intimately interwoven to the cell's own shape, and in particular cell polarity and organization of mechanosensitive and mechanoresisting elements within the cytoskeleton and junctional complexes (Butcher et al. 2009; Jaalouk and Lammerding 2009; Provenzano and Keely 2011). A cell embedded within an epithelial sheet is perfectly adapted to sustain the mechanical properties of its tissue; as such, that cell may keep its shape for as long as the mechanical features of its surroundings remain constant. However, when mechanical stresses cannot be sustained by the existing structures, the cell is forced to rewire its mechanical connections and, as such, its form. For example, mammary epithelial cells cultured on a soft ECM (i.e., reconstituted basement membrane) form spheric sheet ("acini") of growth arrested, polarized, and differentiating cells surrounding a central lumen (Barcellos-Hoff et al. 1989). Progressive stiffening of the ECM leads to disturbed cell polarity, increased cell-ECM adhesion and contractility, and perturbed growth control (Paszek et al. 2005; Weaver et al. 1997). In other words, changes in the mechanical properties of the tumor ECM may initiate a "chain reaction" leading to changes in tissue form and architecture, with ensuing alterations in the sites of cell proliferation, asymmetric vs. symmetric cell division, and differentiation.

6.5.3 Combining Soluble and Unsoluble Cues

The events triggered by increased cellular mechanotransduction present clear analogies with processes typically described in the context of EMT induced by soluble factors, such as TGFβ. This suggests that cells experiencing high mechanical stress, such as tumor cells at the border of a stiff collagen stroma, may represent a sensitized background to the effect of EMT-inducing growth factors. Clearly, TGFβ is a very effective inducer of EMT in cells cultured on plastic, that is, a very stiff substrate (Heldin et al. 2009; Zavadil and Bottinger 2005). Intriguingly, however, when monolayers of cells grown on 2D or 3D molds of defined shapes were challenged with TGFβ, a clear spatial pattern emerged: only cells experiencing high mechanical stresses, that is, those located at edges, sharp curvatures or tips underwent EMT (Gjorevski et al. 2012; Gomez et al. 2010; Lee et al. 2011). Conversely, it is also possible that tumor cells with disturbed apico-basal polarity—by partial EMT or mutation/inactivation of polarity factors, or exposed to high levels of TGFβ—may also display increased sensitivity to mechanical gradients. Perhaps this may explain the exquisite spatial and temporal specificity of EMT induction so far observed in vivo, being transiently induced in one cell, and not in the adjacent cell, for example during wound healing and tumor progression (Gjorevski et al. 2012; Thiery et al. 2009).

6.5.4 YAP and TAZ are Downstream of Mechanical Cues

In light of the above discussion, the discovery that YAP and TAZ are activated not just by loss of polarity, but also by mechanical cues, clearly adds an entirely new dimension to our understanding of normal and pathological tissue biology. YAP and TAZ are indeed regulated by ECM stiffness, cell shape, and cytoskeletal tension (Dupont et al. 2011; Wada et al. 2011). When cells are cultured on stiff ECM, YAP, and TAZ are in the nuclei and induce target gene expression, whereas they are inhibited and relocalized in the cytoplasm in cells cultured on a soft ECM (Dupont et al. 2011). Cell shape can be controlled by seeding cells on microprinted fibronectin "islands" of different sizes (Chen et al. 1997) (Fig. 6.3a). YAP and TAZ are well active in cells with spread cell morphology (as cells growing at low density on plastic dishes or seeded on "big" fibronectin islands), but YAP and TAZ are inactivated in small, roundish, unspread cells seeded on small "islands" (Dupont et al. 2011; Wada et al. 2011) (Fig. 6.3a).

Crucially, YAP and TAZ are the key mediators of the biological effects of ECM stiffness and cell shape. For example, endothelial cells die when forced to remain small, while they proliferate when allowed to spread (Chen et al. 1997). The levels of YAP and TAZ dictate these opposite behaviors: if YAP and TAZ are artificially increased in small cells, these start to proliferate; in contrast, attenuation of YAP and TAZ in spread cells causes them to die (Dupont et al. 2011). Contact inhibition of growth in epithelial cells cultured at high cell density is paralleled by YAP and TAZ inhibition. Although traditionally associated to activation of the Hippo cascade by cell–cell contacts, new evidence suggests that contact inhibition may also be envisioned as consequence of reduced cell shape, due to the confinement of cell-ECM adhesion area (reviewed in (Halder et al. 2012)). A similar type of control applies to non-epithelial, fully mesenchymal cells, such as primary human mesenchymal stem cells (MSC). Analogously to the effects of morphogen gradients, these cells differentiate into distinct histotypes depending on the stiffness of the ECM in which they are cultured (Fig. 6.3b): MSCs become osteoblasts at high stiffness, muscle at intermediate stiffness and neurons or adipocytes on soft ECMs (Engler et al. 2006; McBeath et al. 2004). Again, YAP and TAZ take control of this differentiation: when the high YAP/TAZ levels of stiff MSC is lowered by siRNA-mediated knockdown, the stiff MSC behave as if they were on a soft ECM. Conversely, fates typical of elevated mechanical stimuli can be induced in soft MSC by sustaining YAP and TAZ expression (Dupont et al. 2011).

Interestingly, the regulation of YAP and TAZ by mechanical cues differs from their regulation by EMT, polarity, and the Hippo pathway. Indeed, lowering mechanical cues leads to YAP and TAZ cytoplasmic relocalization and degradation in epithelial, mesenchymal, post-EMT cells, as well as in cells depleted of LATS, or expressing LATS insensitive YAP (Dupont et al. 2011). Cell shape and mechanical cues are intimately associated to the regulation of the Rho family of small GTPase, of ROCK and MLCK, and to corresponding changes in the tensile properties and dynamics of the actomyosin cytoskeleton (Mammoto and Ingber 2009; Parsons et al. 2010;

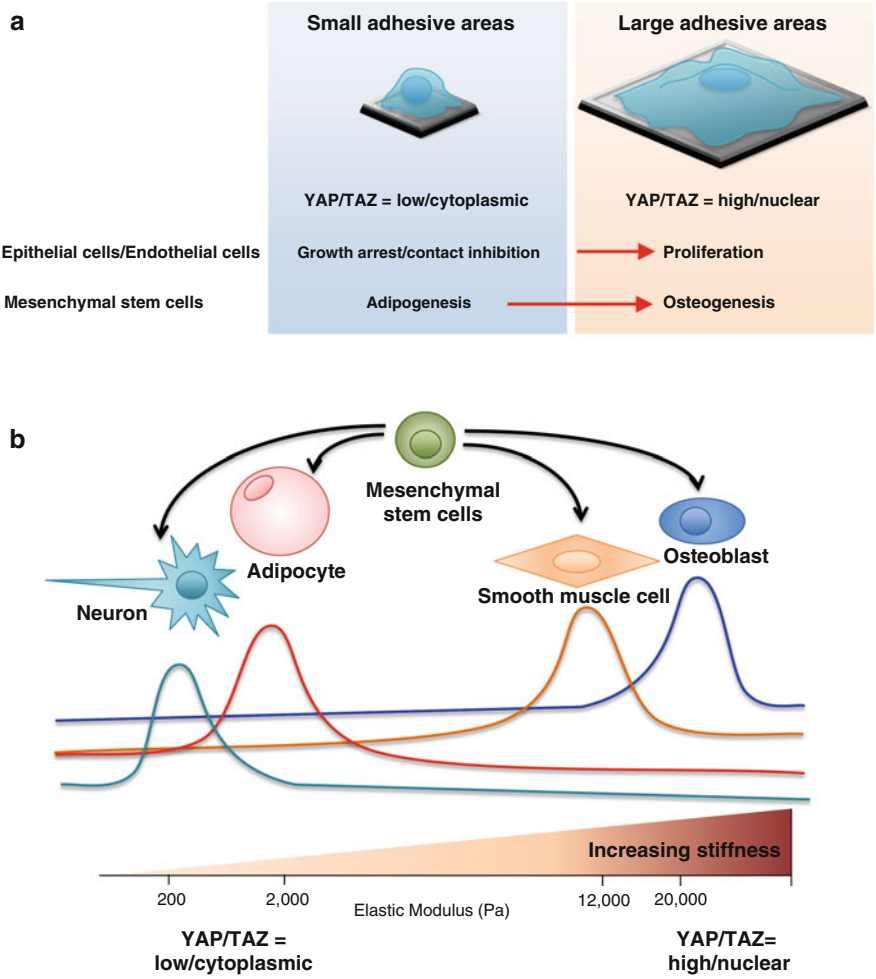

Fig. 6.3 Mechanical and architectural cues inform cell decisions. (**a**) Cell shape can be regulated by controlling the area to which cells can adhere by means of fibronecting microprinting techniques. Cell shape controls YAP and TAZ levels, nuclear vs. cytoplasmic localization and transcriptional activities, as well as a number of biological effects in distinct cellular contexts. YAP and TAZ levels mediate these different behaviors. For example, MSCs cultured in small adhesive areas differentiate as adipocytes whereas they become osteoblasts when cultured in large adhesive areas (McBeath et al. 2004). (**b**) Cell differentiation of pluripotent cells can be directed by ECM elasticity (see text). (*Pa* Pascal, is the tensile strength unit of measurement). YAP and TAZ are nuclear and active in spread cells and in cells experiencing a stiff environment (modified from Halder et al. 2012)

Wozniak and Chen 2009). In line with this notion, YAP and TAZ are dependent on this cytoskeletal pathway for their activity (Dupont et al. 2011; Fernandez et al. 2011; Sansores-Garcia et al. 2011; Wada et al. 2011; Zhao et al. 2012). Effective disruption of the F-actin cytoskeleton causes quantitative inactivation of YAP and TAZ in a manner largely independent from LATS(Dupont et al. 2011). This indicates that

cytoskeletal inputs are simply overarching signals essential for YAP/TAZ activity, and that, probably, other regulations at the level of the Hippo cascade or other inputs may modify or cooperate with, but not completely overrule the information provided by the mechanical context (Halder et al. 2012).

6.6 Concluding Remarks and Future Perspectives

By reviewing the current status of YAP and TAZ signaling, its biological properties and regulation, we realized how many fundamental questions remain unaddressed. Here we just highlight few of them, hoping to inspire new research avenues.

1. What are the targets of TAZ, and possibly YAP, involved in cancer stem cells self-renewal and tumor initiation? How is YAP, and possibly TAZ, regulating the amplification of normal stem cells and progenitor cells in several normal tissues? Is there a universal "stemness" potential conferred to cells by YAP and TAZ? Answering these questions may entail investigating the connections between YAP and TAZ and regulation of symmetric vs. asymmetric cell division
2. How do YAP and TAZ control cell proliferation and survival? These biological traits are at the centerpiece of YAP and TAZ activity in cancer and organ size control. Yet, our knowledge of these processes is still limited
3. What part of the genome is controlled by YAP and TAZ? And what "package" of targets can recapitulate YAP and TAZ biological effects? These studies may reveal new avenues to tackle YAP and/or TAZ activity in basic and applied research
4. Are YAP and TAZ directly regulated by soluble growth factors? Efforts have been dedicated to study how other signaling pathways—including the TGFβ, BMP, Wnt cascade—are modified by the Hippo pathway and YAP or TAZ (Varelas and Wrana 2011). In contrast, a more direct involvement of YAP/TAZ regulation and activity in other pathways has been largely neglected. YAP and TAZ may serve as hub at the crossroad of multiple pathways
5. How are YAP and TAZ regulated by mechanical cues? This represents "the Antartica" of YAP and TAZ biology. Potential mechanisms may include the presence of unknown inhibitors unleashed in soft-cells, or of unknown activators unleashed in stiff cells
6. Are there more kinases, other than MST and LATS, that affect YAP and TAZ activity? Evidences in favor of this hypothesis have surfaced in the characterization of MST1/2 knockout livers, where YAP could be still phosphorylated by a non-LATS kinase (Zhou et al. 2009), and from the analyses of phospho-dependent but LATS-independent YAP regulation by α-catenin in keratinocytes (Schlegelmilch et al. 2011)
7. If YAP and TAZ are such powerful oncogenes, why aren't they directly activated by mutations in human cancers? A plausible explanation may be that YAP and TAZ activity may require, in the *in vivo* microenvironment, the concomitant

presence of several activating inputs, each one *per se* insufficient and not recapitulatable by missense mutations

8. Given the dominant regulation of YAP and TAZ by mechanical cues, can YAP and TAZ activity or localization be used to monitor the spatiotemporal distribution of mechanical cues in vivo?
9. Research in the Hippo field has concentrated the attention on YAP and TAZ regulation in epithelial cells. However, these factors are essential regulators of cell behaviors in fibroblasts and other non-epithelial cells, including those that infiltrate tumors and contribute to their stromal composition. Clearly, YAP and TAZ regulation in these cellular contexts must be as tight as it is in epithelia, but unlikely connected to the polarity regulatory branch. Are mechanical cues the central regulators of YAP and TAZ in these cell types? Connected to these open issues is the fact that fibroblast stiffening has been implicated in tumor progression(Goetz et al. 2011). To what extent is this dependent on cell-autonomous and non-cell-autonomous regulations of YAP or TAZ?
10. Can we identify inhibitors of TAZ activity? These molecules may be ideal candidate as anticancer stem cell therapeutics

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Part III
Apoptosis and Hippo Signaling

Chapter 7
Hippo Pathway and Apoptosis

Garth Hamilton and Eric O'Neill

Abstract The Hippo pathway is an established pathway that regulates apoptosis. The earliest characterisations of the mammalian MST1/2 kinases indicated that they were potent inducers of apoptosis in response to a wide range of stimuli. Elucidation of pathway components via genetic screens in *Drosophila* revealed that signalling through the Hippo pathway is required for the induction of apoptosis during development. Central to control of developmental apoptosis in *Drosophila* is the regulation of the transcriptional co-activator Yki, whose interaction with transcription factors including Sd, Mad and Tsh/Hth drives the transcription of potent apoptotic inhibitors including Diap-1 and the microRNA Bantam. In mammals it is clear that the core MST1/2-LATS1/2 kinase cassette has various downstream components which lead to apoptosis including the transcription of pro-apoptotic target genes via multiple transcription factors, caspase activation and histone modification. The LATS1/2 kinases and YAP function in a complex network with p53 and its associated regulatory proteins from the ASPP family which, through association with YAP, can have opposing effects on apoptosis. While it is clear that YAP is an important inhibitor of apoptosis in mammals and is subject to similar regulation to that of Yki, YAP also promotes the transcription of pro-apoptotic target genes via association with p73. Evidence suggests that the tumour suppressor RASSF1A is an important determinant in mediating YAP pro-apoptotic activities through regulation of YAP transcription factor interactions.

Keywords Hippo • Apoptosis • p53 • p73 • Yap • Caspase

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_7,

7.1 Introduction

Apoptosis, or programmed cell death, is an important process that removes superfluous or damaged cells during development and maintains organism homeostasis (Fuchs and Steller 2011). The Hippo pathway is an evolutionary conserved pathway that regulates tissue size during development by responding to upstream signals generated from cell-cell contacts and spatial development of the organ. This allows the organ to achieve correct cell number and facilitates the removal of excess cells, which are present during developmental stages. Studies from the model organism *Drosophila melanogaster* have demonstrated that the Hippo pathway invokes apoptosis in the developing organism through inhibition of the transcriptional co-activator Yki, which mediates expression of pro-proliferative and anti-apoptotic genes. Close co-ordination and integration of cell death and cell growth shapes the course of tissue development. In *Drosophila* activation of the Hippo pathway is required to inactivate Yki, and failure to do so results in an inability to induce apoptosis in superfluous cells resulting in gross tissue overgrowth (Halder and Johnson 2011; Pan 2010; Staley and Irvine 2012; Zhao et al. 2011).

In the mammalian system, the Hippo pathway and components of the core kinase unit (MST1/2-LATS1/2) are recognised as important regulators of apoptosis. As is the case in *Drosophila*, the mammalian pathway regulates the Yki homologues YAP and TAZ, which also promote the expression of pro-proliferative and anti-apoptotic genes (Huang et al. 2005). However, growing evidence now suggests that in mammals YAP has a dual role and can both induce and inhibit apoptosis. The pro-apoptotic activity of YAP is due to its ability to activate p73 (Strano et al. 2005), a member of the p53 family, and promote expression of pro-apoptotic members of the bcl-2 family (Matallanas et al. 2007). The expression of these proteins commits a cell to apoptosis as they promote mitochondrial permeabilisation, resulting in the release of cytochrome C and the formation of the apoptosome, a scaffolding platform required for caspase activation (Fuchs and Steller 2011). As outlined below, many apoptotic stimuli activate the mammalian Hippo homologues MST1 and MST2, which in turn drive many classical features of apoptosis such as caspase activation, chromatin condensation and DNA fragmentation. Furthermore, it is also becoming apparent that the pro-apoptotic activities of this pathway are opposed by the activity of AKT, a kinase which is frequently linked with the inhibition of apoptosis. It is the aim of this chapter to outline the stimuli, mechanisms and pathways that have been shown to require Hippo pathway members for the induction of apoptosis, with the aim to demonstrate how they may be involved in development, and also how they induce apoptosis in response to extrinsic stimuli.

7.2 The Hippo Pathway and Regulation of Developmental Apoptosis in *Drosophila*

The upstream signalling elements of the Hippo pathway that transmit information regarding cell-cell contact and cell polarity regulate the activity of the Hippo kinase, which in turn regulates the expression of genes that govern apoptosis. A recurring

observation made in the examination of flies with deletions of Hippo pathway components is a failure to induce sufficient apoptosis in the developing tissue, which in part facilitates the tissue overgrowth phenotypes observed. These can range in subtle increase in cell number to massive overgrowth of organs such as the eye or wing depending on the nature of the deletion (Halder and Johnson 2011; Pan 2010; Staley and Irvine 2012). A common mechanism that has emerged from these studies is a failure to inhibit the expression of anti-apoptotic genes whose transcription is promoted by the transcriptional co-activator Yki. Yki itself has no DNA-binding activity but, as outlined below, enhances the activity of several transcription factors which are responsible for driving the transcription of both anti-apoptotic genes and a microRNA that in turn regulates the expression of pro-apoptotic genes.

7.3 The Core Hippo Signalling Module and the Yki-Dependent Inhibition of Apoptosis

All upstream components of the pathway (Fig. 7.1a) converge on the core Hippo signalling module which consists of the Ser/Thr kinases Warts and Hippo, and the scaffold proteins Sav and Mats that enhance their activity. The deletion of either *Wts* (Justice et al. 1995; Xu et al. 1995), *Hpo* (Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003), *Sav* (Harvey et al. 2003; Tapon et al. 2002) or *Mats* (Lai et al. 2005) result in the most severe tissue overgrowth phenotypes described, with accompanying decreases in apoptosis as determined by classical markers of apoptosis including decreased TUNEL staining, decreased activation of the *Drosophila* Caspase-3 like protein DrICE, decreased expression of pro-apoptotic proteins and increased expression of the *Drosophila* inhibitor of apoptosis (IAP) Diap-1. Furthermore, deletion of *Hpo Mats*, *Wts* or *Sav* reduces apoptosis promoted by the overexpression of Hid, Grm or Rpr, which are members of the Reaper Bcl-2 family and are potent pro-apoptotic genes within the intrinsic cell death pathway (Fuchs and Steller 2011; Harvey et al. 2003; Lai et al. 2005; Pantalacci et al. 2003; Tapon et al. 2002). In contrast, the overexpression of Hpo, Sav or Wts, or activation of the pathway by constitutively targeting Mob to the membrane, results in the induction of apoptosis (Ho et al. 2010; Udan et al. 2003; Verghese et al. 2012). The ability of the core Hippo unit to induce apoptosis in *Drosophila* is dependent on the inhibition of the transcriptional co-activator Yki, which when overexpressed results in tissue overgrowth and defective apoptosis, and can even block apoptosis due to overexpression of the Dronc initiator caspase and its co-factor Dark (Huang et al. 2005; Verghese et al. 2012).

Inhibition of Yki transcriptional activity is mediated by Wts-dependent phosphorylation. Wts phosphorylates Yki on multiple sites which share the consensus motif (HX(R/H/K)XX(S/T)) including Ser111, Ser250 and Ser168 (the equivalent of Ser127 in YAP) (Dong et al. 2007; Huang et al. 2005; Oh and Irvine 2008, 2009; Ren et al. 2010; Zhao et al. 2007). Phosphorylation of Yki on Ser168 creates a binding site for 14-3-3 proteins which sequester Yki in the cytoplasm

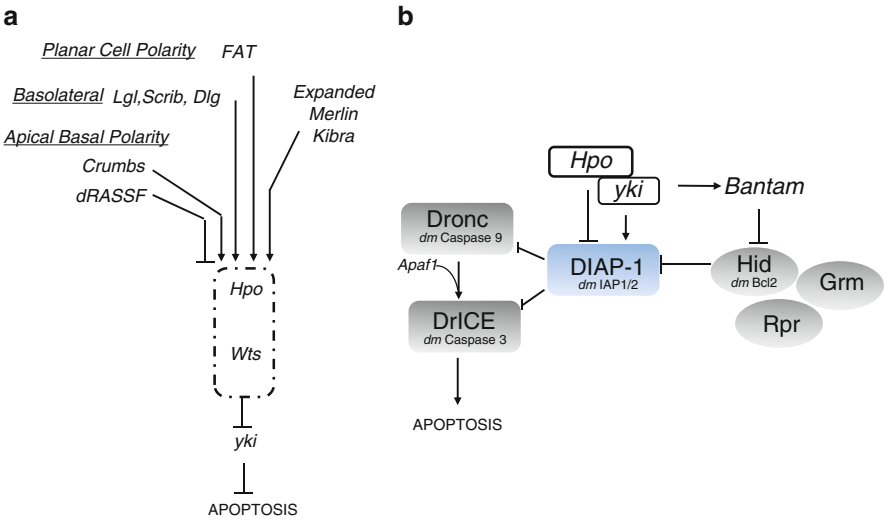

Fig. 7.1 Hippo pathway-mediated regulation of apoptosis in *Drosophila*. (**a**) Multiple upstream signalling inputs including the atypical cadherin FAT, the Expanded-Merlin-Kibra complex, Crumbs and the Lgl, Scrib Dlg complex positively regulate the activity of the core Hippo kinase cassette. dRASSF negatively regulates Hpo activity. Wts phosphorylates Yki and inhibits its activity by promoting relocalisation from the nucleus to the cytoplasm blocking the transcription of anti-apoptotic genes such as Diap-1. (**b**) Yki promotes the transcription of Diap-1 and the microRNA Bantam which protect against apoptosis. Diap-1 inhibits the *Drosophila* caspases Dronc and DrICE and Bantam represses the translation of Hid. Hid is a member of the Bcl2 family (which includes Rpr and Grm) of anti-apoptotic proteins and inhibits Diap-1 binding to Dronc and DrICE

(Dong et al. 2007; Oh and Irvine 2008; Ren et al. 2010) where it is prevented from interacting with its transcription factor (TF)-binding partners including Scalloped (Sd), (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008; Zhao et al. 2008), Homothorax (Hth), Teashirt (Tsh), (Peng et al. 2009) and Mad (Oh and Irvine 2011). The transcriptional output of Yki/Sd, Yki/Tsh/Hth or Yki/Mad complexes is an important determinant in promoting the inhibition of apoptosis in different tissues. However, it has recently been suggested that Yki may have additional roles in inhibiting apoptosis which are independent of the Hippo pathway. Yki has been shown to enhance the anti-apoptotic activity of the non-receptor tyrosine kinase ACK. Although the molecular targets are not defined, this does not require Yki-mediated transcriptional regulation or nuclear localisation (Schoenherr et al. 2012).

7.4 Yki/TF Complexes and the Regulation of Apoptosis

The Yki/Sd complex has been shown to bind to enhancer elements in the *Diap-1* promoter resulting in elevated Diap-1 expression (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008). Indeed, Sd was shown to bind to a minimal 26 bp Hippo response

element that drove Hippo-dependent up-regulation of Diap-1 (Wu et al. 2008). Diap-1 is a member of the IAP family and is an important modulator of apoptosis in developing *Drosophila* tissue (Kornbluth and White 2005; Steller 2008). In the *Drosophila* embryo, loss of Diap-1 induces ubiquitous apoptosis through inappropriate caspase activation (Goyal et al. 2000; Wang et al. 1999). Diap-1 is an E3 ligase that ubiquitinates and inhibits the *Drosophila* initiator caspase Dronc (caspase 9 homologue) via proteasomal degradation (Wilson et al. 2002), and also the effector caspase drICE (Caspase3 homologue) (Ditzel et al. 2008) via a mechanism involving non-degradative polyubiquitination (Fig. 7.1b). Thus, the enhanced Yki-dependent transcription of Diap-1 in Hippo pathway mutants is a potent anti-apoptotic mechanism that facilitates tissue overgrowth. It is interesting to note that the minimal Hippo response element characterised in the Diap-1 promoter contains non-Sd-binding sites that are absolutely required Diap-1 expression, indicating that additional uncharacterised Yki/TF complexes may regulate Diap-1 expression (Wu et al. 2008).

In addition to the regulation of Diap-1, several Yki/TF complexes including Yki/Sd (Zhang et al. 2008), Yki/Hth (Peng et al. 2009) and Yki/Mad (Oh and Irvine 2011) promote the transcription of *Bantam*, a microRNA which regulates cell proliferation and apoptosis (Brennecke et al. 2003). When overexpressed *Bantam* rescues cells from apoptosis induced by overexpression of Hpo, and can promote growth in cells expressing decreased levels of Yki. In contrast, deletion of *Bantam* blocks Yki-driven overgrowth (Nolo et al. 2006; Thompson and Cohen 2006). Although a limited number of *Bantam* microRNA targets have been identified, the pro-apoptotic gene *Hid*, whose expression is repressed by *Bantam* (Fig. 7.1b), appears to have the clearest impact on apoptosis (Brennecke et al. 2003). Notably, Yki mutant clones which have severely reduced Yki expression have elevated expression of Hid (Nolo et al. 2006).

Hid is a member of the Reaper family of Bcl-2 like pro-apoptotic proteins, which also includes Reaper (Rpr), Sickle (Skl) and Grim (Grm). Hid binding via its IBM domain antagonises Diap-1 function by competing with *Drosophila* caspases for binding to the BIR domain of Diap-1, which would otherwise associate with and inhibit caspase activity (Fig. 7.1b). In addition, Hid also promotes the auto-ubiquitination and proteolytic degradation of Diap-1 (Yoo et al. 2002) allowing the removal of this potent caspase inhibitor. Thus, Hid promotes elevated caspase activity and induction of apoptosis (Fuchs and Steller 2011; Kornbluth and White 2005; Steller 2008).

Hpo overexpression has also been reported to specifically induce Hid, resulting in Hid-dependent cell death. Interestingly, Hpo overexpression does not up-regulate the expression of Reaper family members Grm or Rpr, suggesting that the Hpo pathway regulates the expression of a specific subset of proteins that control apoptosis in *Drosophila* (Udan et al. 2003). Given the role of Yki in regulating Hid expression through *Bantam*, it is likely that the elevated Hid expression reported in Hpo overexpressing clones is the result of sustained Yki inhibition. However, it is also possible that Hid expression may be controlled by other Hpo-regulated mechanisms (Fig. 7.1b). To date no other pro-apoptotic targets which are negatively regulated by *Bantam* have been identified.

The activity of Hpo itself has also been suggested as an important regulator of apoptosis in *Drosophila*. Hpo has been reported to phosphorylate Diap-1, resulting in decreased Diap-1 protein stability (Pantalacci et al. 2003). However, overexpression of Hpo within imaginal discs does not appear to have an effect on Diap-1 levels (Verghese et al. 2012). Overexpression of Hpo increases the transcription and activity of Dronc and furthermore, Hpo synergises with Dronc overexpression leading to a more dramatic apoptotic response. In contrast, overexpression of Yki, or deletion of *Wts*, blocks Dronc transcription suggesting that signalling through the Hippo pathway balances the expression and activity of pro- and anti-apoptotic signalling to determine cell fate (Verghese et al. 2012).

Overexpression or deletion of the upstream signalling inputs to the Hippo pathway that provide information on cell-cell contact and cell polarity also influence the induction of apoptosis through the negative regulation of Yki. The Expanded-Merlin-Kirba complex and the recently characterised interacting proteins PEZ and Echinoid (Baumgartner et al. 2010; Bennett and Harvey 2006; Cho et al. 2006; Genevet et al. 2010; Hamaratoglu et al. 2006; Pellock et al. 2007; Poernbacher et al. 2012; Silva et al. 2006; Tyler and Baker 2007; Yu et al. 2010; Yue et al. 2012), Fat and its ligand Dachous (Bennett and Harvey 2006; Cho et al. 2006; Rogulja et al. 2008; Silva et al. 2006; Tyler and Baker 2007; Willecke et al. 2006, 2008), the Lgl, Scrib Dlg complex (Grzeschik et al. 2010), and Crumbs (Chen et al. 2010; Grzeschik et al. 2010; Ling et al. 2010; Robinson et al. 2010) have all been shown to regulate Yki-dependent Diap-1 transcription/expression. However, despite up-regulation Diap-1 in single mutant tissue most large-scale developmental apoptosis proceeds and the observed decrease in apoptosis is often more subtle. This is in contrast to the more dramatic effect of the core Hippo cassette which is universally required to induce apoptosis in the tissues examined to date. The discrepancy may be that the level of Diap-1 induced is insufficient to protect against apoptosis or as recently reviewed (Halder and Johnson 2011; Pan 2010; Staley and Irvine 2012), the role of each of these upstream complexes in regulating developmentally controlled apoptosis is complicated by the observations that these upstream elements function with partial redundancy in different tissues and are often required at different developmental stages (Milton et al. 2010). Thus fine tuning and the strength of signal from upstream elements to induce apoptosis in an Hpo-dependent manner is also tissue specific and developmentally regulated.

The literature suggests that the role of Yki transcription factor complexes in the regulation of developmentally driven apoptosis is tissue specific (Staley and Irvine 2012). Indeed, the Yki-binding partners Sd (Goulev et al. 2008) and Hth/Tsh (Peng et al. 2009) have distinct predominant roles in the wing disc and anterior eye disc respectively. In contrast, it has been suggested that Mad serves as a general TF for Yki in the maintenance of growth control as it is ubiquitously expressed (Oh and Irvine 2011). This regulated selection of transcription factor use presumably allows the expression of a specific subset of genes in a tissue-specific manner and reflects the input from additional signal pathways in order for faithful organ development through co-ordinated spatial growth and apoptosis. It will thus be of interest to determine if there are further anti-apoptotic targets regulated by Yki in a

tissue-specific manner or indeed if other Yki transcription factor complexes contribute to the anti-apoptotic transcription profile of Yki.

7.5 Regulation of Apoptosis in Mammalian Cells by the MST-LATS Kinase Cassette

7.5.1 Regulation of Apoptosis via the MST1 and MST2 Kinases

The mammalian orthologues of the Hippo kinase are MST1 and MST2, themselves named as the mammalian homologues of the yeast Sterile Twenty kinase (Creasy and Chernoff 1995a, b; Taylor et al. 1996). Prior to the recognition of Hpo in developmental models, MST1 and MST2 have long been recognised as pro-apoptotic kinases that induce apoptosis (Fig. 7.2) in response to a range of stimuli including sodium arsenite, etoposide, cyclohexamide, staurospaurine, H_2O_2, FAS, TNFα cytotrienin A, and bisphonates (Graves et al. 1998; Kakeya et al. 1998; Lee et al. 2001; Reszka et al. 1999; Taylor et al. 1996). Furthermore, MST1/2 are required for apoptosis induced by constitutively active KRasV12 and function in a tumour suppressive apoptotic pathway (Khokhlatchev et al. 2002; Matallanas et al. 2011). In vivo experiments with mice harbouring genetic deletions of both *MST1* and *MST2* demonstrated that cells within the liver of mice were more resistant to TNFα and FAS-induced apoptosis (Song et al. 2010; Zhou et al. 2009). Furthermore, MST1 has also been reported to mediate cardiomyocyte apoptosis in the heart in response to tissue damage induced by various insults including myocardial infarction, ischemic reperfusion injury and chronic pressure overload (Del Re et al. 2010; Odashima et al. 2007; Yamamoto et al. 2003), further highlighting the pro-apoptotic role of MST1/2 in response to a variety of stimuli.

In response to apoptotic stimuli MST1/2 are cleaved by caspases which removes a C-terminal auto-inhibitory domain and the resulting kinase domain displays greatly enhanced activity and altered substrate specificity (Figs. 7.2 and 7.3) (Anand et al. 2008; Graves et al. 1998, 2001; Lee et al. 1998, 2001). MST1 is cleaved by caspase 3 and 7 at Asp326 and Asp349, whereas MST2 is cleaved at Asp322 (Song and Lee 2008). Although the Asp residues corresponding to 326/322 in MST1/2 are evolutionary conserved between MST1/2 homologues in mammals, *Drosophila* and nematodes (Lee et al. 2001), residues C-terminal to the Asp influence MST cleavage. In mammals this Asp residue is flanked by an Ser (Ser327) which has been identified as a potential MST1/2 autophosphorylation site (Glantschnig et al. 2002; Graves et al. 2001) and mutation to Asp inhibits caspase 3-mediated cleavage of MST1 (Glantschnig et al. 2002; Graves et al. 2001). In contrast to MST1/2, Hippo contains a flanking Asp residue which is thought to prevent Hippo from being cleaved by DrICE (Wu et al. 2003). Thus this may be an evolutionary difference that allows a distinction to be made between apoptotic and non-apoptotic functions of MST1/2 in the cell. Overexpression of MST1 has also been demonstrated to induce

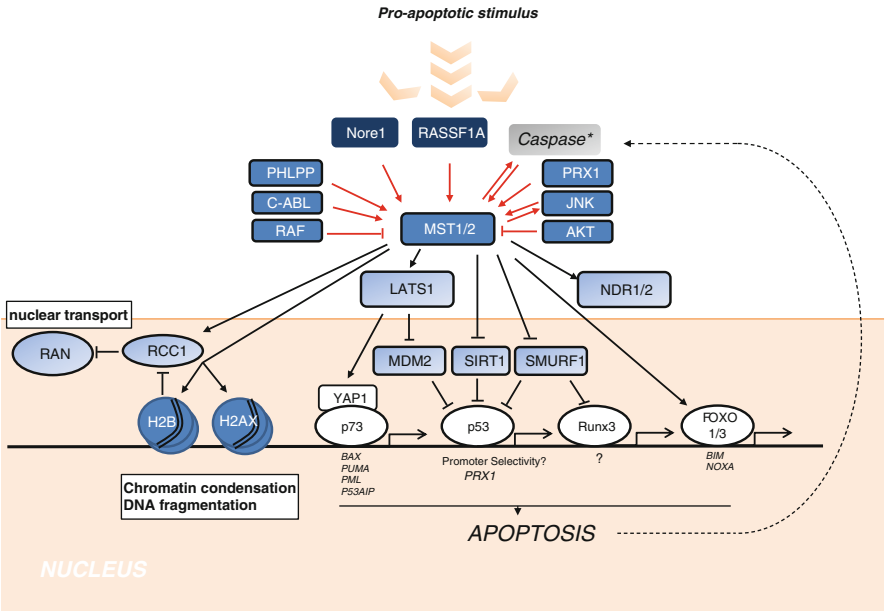

Fig. 7.2 Multiple pathways of MST1/2-mediated apoptosis. MST1/2 regulate multiple apoptotic pathways in response to wide range of pro-apoptotic stimuli (see text for details). MST1/2 activity is regulated by the RASSF family of scaffold proteins and by caspase activation. Additionally, phosphorylation by other kinases regulates MST1/2 activity. MST1 phosphorylates histones H2B and H2AX, which promotes chromatin condensation and DNA fragmentation. Phospho H2B also binds and sequesters RCC1 and RAN-GTP onto chromatin-blocking nuclear transport. Transcription of pro-apoptotic genes is also promoted by MST in response to apoptotic stimuli. Activation of MST1/2 also activates a number of downstream kinases which have been associated with the induction of apoptosis although the targets of some of these kinases remain to be identified

caspase 3 and 8 activity in a JNK-dependent manner, suggesting that MST can also directly activate caspase activity suggesting a positive feedback loop (Fig. 7.2) (Ura et al. 2001b). Caspase-mediated cleavage of MST1/2 also removes two C-terminal nuclear export sequences allowing the truncated kinase domain to traffic to the nucleus resulting in enhanced nucleosomal DNA fragmentation, nuclear condensation and membrane blebbing, all of which are hallmarks of apoptosis (Lee et al. 2001; Lin et al. 2002; Ura et al. 2001a).

It is interesting to note that in quiescent tissue, caspase-mediated cleavage of MST has a function independent of apoptosis (Zhou et al. 2009). In the murine liver MST1/2 are present almost exclusively as the truncated N-terminal kinase domain. Previous studies demonstrated that a truncated species of MST2 was detected in quiescent cells (Wang and Fecteau 2000), and that caspase 3-mediated cleavage of MST2 was required for muscle differentiation (Fernando et al. 2002). Caspases are now being recognised as having many non-apoptotic roles (Fuchs and Steller 2011) and thus it will be of interest to determine what MST-regulated substrates are contributing to maintenance of quiescence.

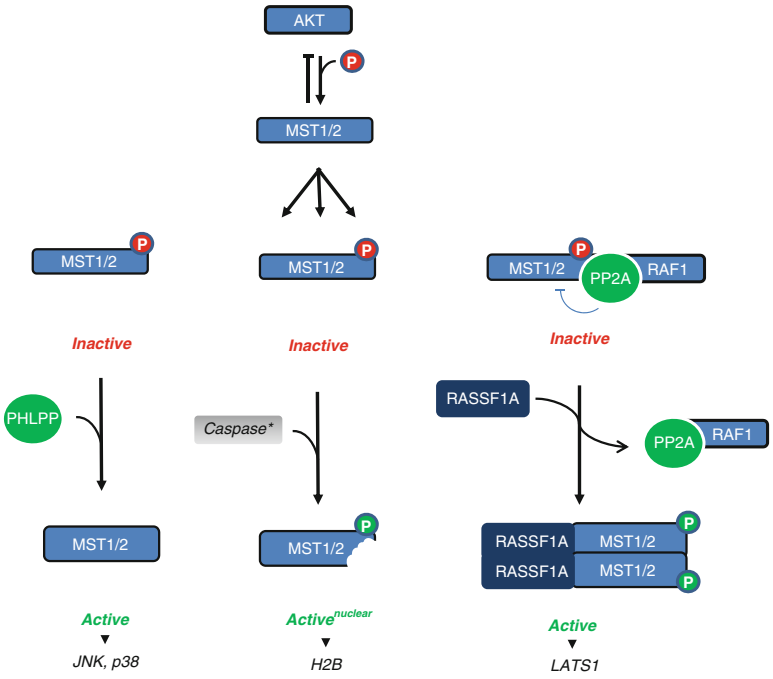

Fig. 7.3 Negative regulation of MST activity and apoptosis. The activity of MST1/2 and AKT acts antagonistically. MST1/2 inhibit AKT and MST activity is negatively regulated by AKT. AKT phosphorylates MST1 on Thr120 and Thr 387 and MST2 on Thr117 and Thr384. Phosphorylation on these sites promotes association with Raf1 which sequesters MST1/2 into an inhibitory complex, blocks MST1/2 dimerisation which is required for autophosphorylation and caspase-mediated cleavage and activation of MST1/2. The inhibition of MST1 by AKT is opposed by the PHLPP phosphatase. RASSF1A disrupts the RAF1/MST1/2 inhibitory complex, prevents PP2A-mediated dephosphorylation of MST1/2 and promotes MST1/2 dimerisation. Phosphorylation of MST1/2 has been shown to inhibit a number of pro-apoptotic pathways

In addition to the caspase-mediated activation of MST1/2, the best characterised positive upstream regulators of the MST kinases are the RASSF family of scaffold proteins, in particular RASSF1A and NORE1 (Fig. 7.2). RASSF1A and NORE1 have both been shown to enhance MST1/2 activity and apoptosis following oxidative stress, treatment with TNF, FAS or oncogenic RAS and RASSF1A is required to activate the downstream targets of MST1/2, NDR and LATS1/2 (Khokhlatchev et al. 2002; Matallanas et al. 2007; Oh et al. 2006; Park et al. 2010; Vichalkovski et al. 2008; Yuan et al. 2009). Furthermore *Nore1*$^{-/-}$ MEFs are resistant to TNF-induced activation of MST1 and apoptosis, and display reduced activation of JNK and p38 MAP kinase. Interestingly NORE1 only enhances MST activity in response to a limited number of stimuli (Park et al. 2010). Both RASSF1A and NORE1 promote MST1/2 dimerisation and auto-activation which is thought to be required before caspase-mediated cleavage can occur (Matallanas et al. 2007; O'Neill et al. 2004; Praskova et al. 2004; Romano et al. 2010).

7.6 Downstream Effectors of the MST1/2 Kinases

Overexpression of MST1 has been shown to result in apoptosis with accompanying activation of the stress-activated MAP kinases, JNK and p38 MAP kinase (Fig. 7.2) (Glantschnig et al. 2002; Graves et al. 1998; Qiao et al. 2010; Ura et al. 2007). Indeed *MST1*$^{-/-}$, *MST2*$^{-/-}$ null MEFs display reduced JNK activation following TNFα stimulation (Song et al. 2010), suggesting that JNK is natural downstream target of MST1/2 in response to death receptor signalling. Although dependent on the upstream JNK activators MKK4 and MKK7 (Ura et al. 2007), the mechanism as to how MST1 activates JNK or p38 has not been defined. As outlined below, JNK signalling is important in MST1-induced apoptosis mediated by the FOXO transcription factors. Furthermore, JNK activation also contributes to MST1 activation. JNK phosphorylates MST1 on Ser82 and mutation of this site, or treatment with JNK inhibitors, restricts MST1 activation, caspase-mediated cleavage and nuclear translocation (Bi et al. 2010).

An additional target of MST1 is the histone H2B, which is phosphorylated on Ser14 in response to a number of stimuli (Fig. 7.2) (Cheung et al. 2003; Teraishi et al. 2006; Wong et al. 2009; Yun et al. 2011). Phosphorylation acts as a trigger for DNA fragmentation during apoptosis which is a biochemical hallmark of late apoptosis. Indeed phospho-Ser14-H2B is abundant in degraded chromatin (Ajiro et al. 2010). Phosphorylation of Ser14 occurs with similar kinetics to that of MST1 cleavage during apoptosis (Cheung et al. 2003) and interestingly, H2B is a better substrate for cleaved MST1 compared to full length (Anand et al. 2008), suggesting that cleavage of MST1 may aid in committing a cell to apoptosis. Additionally, cleaved MST1-mediated phosphorylation of H2B also sequesters RanGTP and its associated GTP-activating protein, RCC1, on the chromatin, collapsing the RanGTP-RanGDP gradient (Fig. 7.2), which is required for the import of nuclear proteins (Wong et al. 2009). Phosphorylation of H2B is evolutionary conserved, and yeast homologue ste20 also phosphorylates H2B at the equivalent residue (Ser10) (Ahn et al. 2005). In addition, MST1 also phosphorylates the H2A variant H2AX on Ser139 (Fig. 7.2) in response to apoptotic inducing stimuli (Teraishi et al. 2006; Wen et al. 2010). This occurs with similar kinetics to caspase 3 cleavage of MST1 and is required for chromatin condensation and DNA fragmentation. These studies would suggest that MST1 kinase activity can commit the cell to apoptosis through modulation of histone and chromatin structure which leads to DNA fragmentation.

7.7 MST1/2-Mediated Transcriptional Regulation of Pro-apoptotic Target Genes

MST1 induces apoptosis in neuronal cells through the regulation of FOXO transcription factors (Fig. 7.2). This is also a conserved pathway as the *Caenorhabditis elegans* MST orthologue cst1 also regulates the activity of the FOXO orthologue

daf-16 (Lehtinen et al. 2006). FOXO transcription factors are inhibited via AKT-mediated phosphorylation, which promotes 14-3-3 proteins-mediated sequestration in the cytoplasm, preventing up-regulation of pro-apoptotic genes or genes involved in cell cycle arrest (Greer and Brunet 2008). MST1 phosphorylates FOXO3 on Ser207 and FOXO1 on Ser212, within the forkhead domain, which blocks 14-3-3 binding and promotes the nuclear translocation and accumulation of FOXO1/3, leading to transcription of the pro-apoptotic proteins BIM and NOXA (Lehtinen et al. 2006; Valis et al. 2011; Yuan et al. 2009). The pro-apoptotic activities of FOXO transcription factors are also positively regulated by JNK in response to oxidative stress (Greer and Brunet 2008) and as JNK phosphorylation of MST1 on Ser82 enhances FOXO3 Ser207 phosphorylation (Bi et al. 2010) these two kinases act co-operatively to induce FOXO-mediated transcription of pro-apoptotic genes (Fig. 7.2). Additionally, inhibition of MST1 polyubiquitination and proteasomal degradation by c-ABL phosphorylation of Tyr433 enhanced FOXO3-mediated BIM expression (Xiao et al. 2011). This site is not conserved on MST2 and may represent a specific route of MST1 activation. Interestingly, MST1 regulation of FOXO in mammals is not always pro-apoptotic. In naive T-cells MST1 is required to protect T-cells from elevated levels of reactive oxygen species (ROS) through FOXO-mediated transcription of ROS detoxifying enzymes, catalase and SOD2 (Choi et al. 2009). Thus MST1-mediated regulation of FOXO transcriptional output may be cell or stress dependent and it remains to be determined what other signalling inputs determine cell fate through FOXO transcriptional output.

MST1 also affects the activity of p53 and induces apoptosis in a p53-dependant manner. In response to DNA-damaging agents MST1 phosphorylates SIRT1, an NAD^+-dependent deacetylase, which antagonises p53 activity (Fig. 7.2). Overexpression of MST1 inhibits SIRT1-dependent p53 acetylation, as well as that of FOXO3, resulting in increased p53 transcriptional activity and cell death (Yuan et al. 2011). P53 also indirectly activates MST1 and drives MST1-induced apoptosis. In response to oxidative stress Peroxiredoxin (PRX1) oligomerises in a p53-dependent manner and is a key intermediate in the induction of MST1 activity (Morinaka et al. 2011). In light of MST1-mediated activation of p53, this may represent a feedback mechanism for amplification of MST1 activity (Fig. 7.2). Additionally, MST1 may activate p53 via other scaffold proteins such as death-associated protein 4 (DAP4) which has been reported to mediate p53-dependent MST1-induced apoptosis (Lin et al. 2002). DAP4 induces MST1 nuclear translocation and was shown to bind p53 directly. One possibility may be that DAP4 helps to scaffold MST1 to other p53-interacting proteins such as SIRT1, although the molecular details of how DAP4 induces MST1-mediated apoptosis are unclear.

A complex of MST2 and WW45 has also been shown to mediate RUNX3-induced cell death (Min et al. 2012). MST2 phosphorylates RUNX3 on Ser17, Thr16, Ser71, Ser77 and Ser81 within the Runt domain. MST2 phosphorylation of RUNX3 promotes translocation to the nucleus by preventing association of the E3 ligase, SMURF1, which otherwise targets RUNX3 for degradation. The targets of MST2/RUNX3-mediated cell death remain to be identified (Fig. 7.2), although it is interesting to note that RUNX3 cooperates with FOXO3 to induce apoptosis and as

such the MST kinases may coordinate the output of multiple transcription factors in order to commit the cell to apoptosis. (Yamamura et al. 2006). The MST2/RUNX3 interaction is conserved in *Drosophila* (Min et al. 2012) and it will be interesting to determine if this is involved in Hippo pathway-mediated developmental apoptosis.

7.8 Negative Regulation of MST1/2-Mediated Apoptosis

As MST1/2 are pro-apoptotic kinases, their activity must be regulated in order to prevent inappropriate cell death. The mechanisms reported to regulate MST1/2 activity include dephosphorylation, sequestration into inhibitory complexes, phosphorylation via AKT, binding to heat shock proteins and down-regulation via microRNA (Fig. 7.3).

Inhibitory complexes with RAF-1 or A-RAF prevent MST2 dimerisation and auto-activation as well as activation of the downstream effectors p38 and JNK (Matallanas et al. 2007; O'Neill et al. 2004; Rauch et al. 2010; Romano et al. 2010). Indeed knockdown of MST2 rescues *Raf-1* null cells from apoptotic stimuli such as FAS and in normal cells it is the RASSF1A-dependent disruption of RAF1 complexes which promotes MST2 dimerisation and auto-activation (Fig. 7.3) (Matallanas et al. 2007; O'Neill et al. 2004; Romano et al. 2010). Early studies demonstrated that binding of MST1/2 to RAF1 recruited the PP2A phosphatase to this complex which dephosphorylated and inactivated MST2 (O'Neill et al. 2004). Recently it has been shown that RASSF1A protects MST1/2 from PP2A-mediated inhibition through dephosphorylation of Thr183/180 (Guo et al. 2011) and is likely to occur through dissociation of the RAF1-MST complex (Fig. 7.3). In an analogous fashion, Hpo is also negatively regulated by dephosphorylation (Ribeiro et al. 2010). The association of MST2 with RAF1 is also disrupted by c-ABL phosphorylation of MST2 on Tyr81 which in turn promotes MST2 dimerisation and auto-activation (Liu et al. 2012).

AKT is a negative regulator of apoptosis (Duronio 2008) and inhibits MST1/2 activity. Growth factor receptor signalling which activates AKT has been shown to inhibit MST1/2 (Fig. 7.3) (Creasy and Chernoff 1995a; Jang et al. 2007; Kim et al. 2010; Matallanas et al. 2011; Romano et al. 2010). AKT phosphorylates MST1/2 on two sites within the canonical AKT substrate recognition motif RXRXXS/T; MST1 is phosphorylated on Thr120 (Yuan et al. 2010b) and Thr387 (Jang et al. 2007) and MST2 is phosphorylated on Thr117 and Thr384 (Kim et al. 2010; Romano et al. 2010). AKT-mediated phosphorylation of MST1 inhibits MST1 kinase activity, FOXO3 Ser207 phosphorylation and caspase-mediated MST1/2 cleavage. Furthermore, autocrine signalling via EGFR and activation of AKT inhibit MST2-mediated LATS1 activation and induction of p53-dependent apoptosis (Matallanas et al. 2011). In addition, phosphorylation of MST2 by AKT also increases with association of MST with the negative regulator, RAF-1 (Romano et al. 2010). Interestingly, Thr387 is also dephosphorylated by the tumour suppressor pleckstrin homology domain leucine-rich repeat protein

phosphatase (PHLPP) which also antagonises AKT activity (Fig. 7.3) (O'Neill et al. 2012). MST1, but not MST2, interacts with PHLPP and dephosphorylation of MST1 phospho-Thr387 by PHLPP increases MST1, p38 and JNK activity with accompanying increases in apoptosis (Qiao et al. 2010). MST1/2 have also been reported to be direct inhibitors of AKT1 (Fig. 7.3) (Cinar et al. 2007). The antagonistic relations relation between MST and AKT is also conserved in *Drosophila,* and Hpo inhibits Yki-mediated expression of AKT (Ye et al. 2012). Recently MST2 was shown to be a target of the tumour-promoting micoRNA 133b which promotes elevated AKT1 and ERK activity (Qin et al. 2012). Thus a complex network exists whereby signalling via MST1/2 and AKT can balance the output of each other in order to determine cell fate. MST1 activity is also regulated by the heat shock protein Hsp70 (Ren et al. 2008). Hsp70-mediated inhibition of MST1 activity is dependent on the proteasomal degradation of MST1 and requires the E3 ligase CHIP for targeting MST1 to the proteasome.

7.9 LATS1/2-Mediated Induction of Apoptosis

Overexpression of LATS1/2 has been shown to induce apoptosis with reported increases in p53 and Bax expression as well as caspase activation and down-regulation of the anti-apoptotic proteins BCL2 and BCL_{xl} (Ke et al. 2004; Xia et al. 2002; Yang et al. 2001). Furthermore, overexpression of LATS2 increased the processing and activation of caspase 9 (Ke et al. 2004). As important regulators of mitosis and the spindle checkpoint (Visser and Yang 2010), LATS1/2 can induce apoptosis in response to failure to satisfy the spindle checkpoint or faithful segregation of the genome. LATS1 has been shown to induce apoptosis in response to spindle damage mediated by the microtubule poison nocodazole (Iida et al. 2004) and LATS2 promotes apoptosis of cells with polyploidy genomes (Aylon et al. 2009, 2010).

The kinase activity of LATS1 is required for the activation of proteins that drive processing and initiation of apoptotic pathways. The C-terminus of LATS1 binds to the PDZ domain of Omi/HtrA2 when it is released from the mitochondria, leading to the processing and activation of this protease in a kinase-dependant manner (Kuninaka et al. 2005). LATS2 does not interact with Omi/HtrA2 (Kuninaka et al. 2007). Omi/HtrA2 is an inhibitor of mammalian IAPS including XIAP and cIAP and binds to IAPs via the IBM domain. Omi/HtrA2 induces degradation of the mammalian IAPs and hence leads to caspase activation (Vande Walle et al. 2008). Depletion of LATS1 leads to elevated IAP expression (Kuninaka et al. 2005). Thus in an analogous manner to the Hippo pathway in *Drosophila*, inhibition of IAP activity is an important downstream target in mammalian Hippo-regulated apoptosis. Interestingly, LATS1 is also an Omi/HtrA2 substrate and Omi/HtrA2 was shown to mediate LATS1 control of the G1/S checkpoint (Kuninaka et al. 2007). It would therefore be interesting to determine if this is also negative feedback mechanism that regulates LATS1-induced apoptosis.

7.10 YAP and TAZ Apoptotic Target Genes and Their Regulation

The LATS1/2-mediated inhibition of YAP transcriptional activity is conserved in mammals. In addition, LATS1/2 also negatively regulates the activity of the YAP-related protein TAZ, which can also promote the transcription of many genes regulated by YAP via a common interaction with the transcription factor TEAD (Zhang et al. 2009; Zhao et al. 2008). LATS1/2 inhibits YAP/TAZ activity in an analogous manner to Wts-mediated inhibition of Yki. LATS1/2 phosphorylates YAP on Ser127 (also Ser61, 104, 164 and 381) and TAZ on Ser89 (also Ser66, 117 and 311) which are within the HX(R/H/K)XX(S/T) LATS consensus motif. Phosphorylation of YAP-Ser127 or TAZ-Ser89 promotes 14-3-3 binding and relocation to the cytoplasm (Dong et al. 2007; Lei et al. 2008; Zhao et al. 2007).

Like Yki, YAP up-regulates the expression of anti-apoptotic genes including the mammalian IAPs BIRC5, BIRC2 and the Bcl2 family member MCL1 (Dong et al. 2007; Liu-Chittenden et al. 2012; Zhao et al. 2008), and down-regulates the expression of the pro-apoptotic proteins BIM and BAX (Vigneron et al. 2010; Zhao et al. 2008). Microarray analysis suggests TAZ can also up-regulate the expression of BIRC5 (Zhang et al. 2009). Furthermore, YAP and TAZ can also protect the cell against anoikis, and LATS-mediated inactivation of YAP helps drive this cell death pathway (Vigneron et al. 2010; Zhao et al. 2012). In a transgenic mouse model, inducible overexpression of YAP was shown to protect the liver from FAS receptor agonist-induced apoptosis, and YAP-dependent BIRC5 expression enhanced colony growth in soft agar (Dong et al. 2007; Liu-Chittenden et al. 2012). In contrast, genetic deletion of *Yap1* in the liver results in increased hepatocyte apoptosis (Zhang et al. 2010). BIRC2 and BIRC5 expression and YAP1 nuclear localisation are enhanced in the heart when WW45 is conditionally deleted (Heallen et al. 2011). However, despite the increase in the expression of these two genes, there is no difference in apoptosis compared to the control mice, suggesting that the ability of YAP to protect against apoptosis may be tissue/context dependant. Expression of constitutively active YAP-S127A in the skin protects keratinocytes from apoptosis (Zhang et al. 2011a), and loss of WW45 in epithelial tissue results in decreased apoptosis within the developing mouse as a result of elevated YAP activity (Lee et al. 2008). In addition, genetic deletion of NF2 in the liver which lies upstream of YAP promotes elevated YAP nuclear localisation and the enhanced transcription of BIRC5 (Liu-Chittenden et al. 2012; Zhang et al. 2010).

Although many YAP-interacting transcription factors have been identified (Pan 2010; Zhao et al. 2011), the YAP/TEAD complex is the best characterised (Zhao et al. 2008). In both human and mouse cells, overexpression of TAZ and YAP induces BIRC5 expression (Zhang et al. 2009; Zhao et al. 2008). YAP/TEAD is found on the promoters of BIRC family members such as BIRC7 and likely mediates the inhibition of BIM expression (Vigneron et al. 2010; Zhao et al. 2008). Indeed the role of TEAD transcription factors in mediating BIRC5 transcription was demonstrated in a recent study where expression of a dominant negative version of TEAD

blocked BIRC5 transcription and enhanced apoptosis in response to treatment with a FAS agonist antibody (Liu-Chittenden et al. 2012).

In contrast to Wts, LATS1/2 has an additional mode of YAP inhibition. LATS1/2 phosphorylation of Ser381 primes YAP for an additional phosphorylation by CK1 δ/ε which in turn recruits the E3 ubiquitin ligase SCFβ-TRCP, leading to ubiquitin-dependent proteasomal degradation (Zhao et al. 2010). A similar mechanism exists for TAZ which requires LATS2-mediated phosphorylation on Ser311 (Liu et al. 2010).

7.11 A Complex Relationship Between LATS, Yap and the p53 Family of Tumour Suppressors

Unlike Yki, YAP activity is not strictly anti-apoptotic and pro-proliferative. Indeed growing evidence suggests that YAP contributes to the induction of apoptosis and that this phenotype is mediated by the p53 family of tumour suppressors namely p63 and p73. Furthermore, proteins which regulate p53 have also been reported to regulate YAP activity. As discussed below, LATS2 is also a p53 target gene and contributes to enhanced p53 activity, suggesting that in mammalian cells a complex network exists between the Hippo pathway the pro-apoptotic activities of p53 and its related family members.

7.11.1 LATS1/2 and p53

The LATS1/2 kinases have been identified as positive regulators of p53, a tumour suppressor with potent pro-apoptotic activity (Vousden and Prives 2009). In response to oncogenically activated K-RAS or H-RAS, both LATS1 and LATS2 have been shown to induce p53 stabilisation and transcription of pro-apoptotic target genes, including p21, BAX, PIG3 and inhibition of BIRC3 expression (Aylon et al. 2006, 2009, 2010; Matallanas et al. 2011). Both LATS1 (Matallanas et al. 2011) and LATS2 (Aylon et al. 2006) promote p53 stabilisation by binding and inhibiting the E3 ligase MDM2, which targets p53 for ubiquitination and proteasomal degradation. Oncogenic K-RAS-driven activation of LATS1 and p53 is mediated by RASSF1A and MST2. In colorectal cancer, apoptosis induced by K-RAS-driven MST2 and LATS1 activation creates a selective pressure to bypass this apoptotic pathway through inactivation of MST2 expression (Matallanas et al. 2011).

LATS2-mediated activation of p53 induces apoptosis in response to a limited range of stimuli that induce genomic instability and the formation of polyploidy genomes (Aylon et al. 2006, 2009, 2010). The selective LATS2-dependent apoptotic response of polyploidy cells requires p53 and as discussed below is promoted by ASSP1, a known p53 regulator (Fig. 7.4). LATS2-mediated activation of p53

removes polyploidy cells through cell cycle checkpoint activation and the induction of pro-apoptotic p53 target genes including BAX, and down-regulation of the apoptotic inhibitor BIRC3 (Aylon et al. 2009, 2010). Interestingly LATS2 is itself a p53 target gene (Aylon et al. 2006; Kostic and Shaw 2000), creating a positive feedback loop between these two tumour suppressors.

The activation of p53 by LATS2 in response to oncogenic H-RAS requires the translocation of LATS2 from the cytoplasm to the nucleus in an ATR-CHK1-dependant manner (Aylon et al. 2009). In response to oncogenic H-RAS, ASPP1 undergoes a LATS2-induced phosphorylation event. Interestingly, LATS1 also interacts with ASPP1 (Vigneron et al. 2010). ASSP1 is a member of the ankyrin-repeat-, SH3-domain- and proline-rich-region-containing family of proteins which specifically regulates p53 apoptotic activity (Sullivan and Lu 2007). Phosphorylation of ASPP1 by LATS2 promotes the cytoplasmic to nuclear translocation of ASPP1, and the ASPP1-LATS2 complex enhances p53 recruitment onto the promoters of pro-apoptotic genes including BAX, CD95, PUMA and GADD45a while reducing p53 recruitment onto non-apoptotic p53 targets such as p21 (Aylon et al. 2010) (Fig. 7.4).

Interestingly ASSP1-LATS2-induced apoptosis is inhibited by YAP. YAP binds to both ASSP1 (Aylon et al. 2010; Vigneron et al. 2010) and ASSP2 (Espanel and Sudol 2001), and YAP antagonises LATS2-ASPP1-induced apoptosis in response to constitutively active H-RAS. YAP inhibits LATS2-ASPP1 translocation to the nucleus through competition with LATS2 for binding to ASPP1 (Aylon et al. 2010) (Fig. 7.4). Furthermore, ASSP1 and YAP also exhibit anti-apoptotic activity in a mechanism which is dependent on the cellular localisation of ASSP1 (Vigneron et al. 2010). In cell lines where ASPP1 is predominately cytoplasmic, ASPP1 contributes to the transcriptional output of YAP. ASPP1 competes with LATS1 for association with YAP, preventing LATS1-mediated phosphorylation of YAP, resulting in increased YAP stability and accumulation in the nucleus. Perhaps the observed increased YAP stability and decreased YAP-S127 phosphorylation is due to ASPP1-dependent phosphatase recruitment. An analogous mechanism has been described for ASPP2-PP1-mediated dephosphorylation of Ser89 in TAZ, and PP1A-mediated dephosphorylation of YAP has been demonstrated to positively regulate YAP activity (Liu et al. 2011; Schlegelmilch et al. 2011) (Fig. 7.4).

The anti-apoptotic phenotype mediated by ASPP1 and YAP is dependent on repression of *Bim* and knockdown of either ASPP1 or YAP enhances apoptosis in response to hydroxyurea treatment. Overexpression of ASPP1 and YAP antagonises p53 activity, inhibiting the expression of p53 pro-apoptotic target genes including, BAX, PUMA and PHLDA3 (Vigneron and Vousden 2012). LATS2 expression is repressed by cytoplasmic ASPP1, as ASPP1 enhances YAP recruitment to the *LATS2* promoter (Fig. 7.4). The enhanced YAP stability and activity conferred by cytoplasmic ASPP1 (Vigneron et al. 2010), coupled with the inhibition of LATS2 expression by YAP (Vigneron and Vousden 2012), indirectly promotes p53 inhibition by preventing LATS2 recruitment to p53 target genes, which when overexpressed with ASPP1 was shown to enhance the expression of pro-apoptotic p53 targets (Aylon et al. 2010) (Fig. 7.4). Unanswered questions remain as to what additional proteins

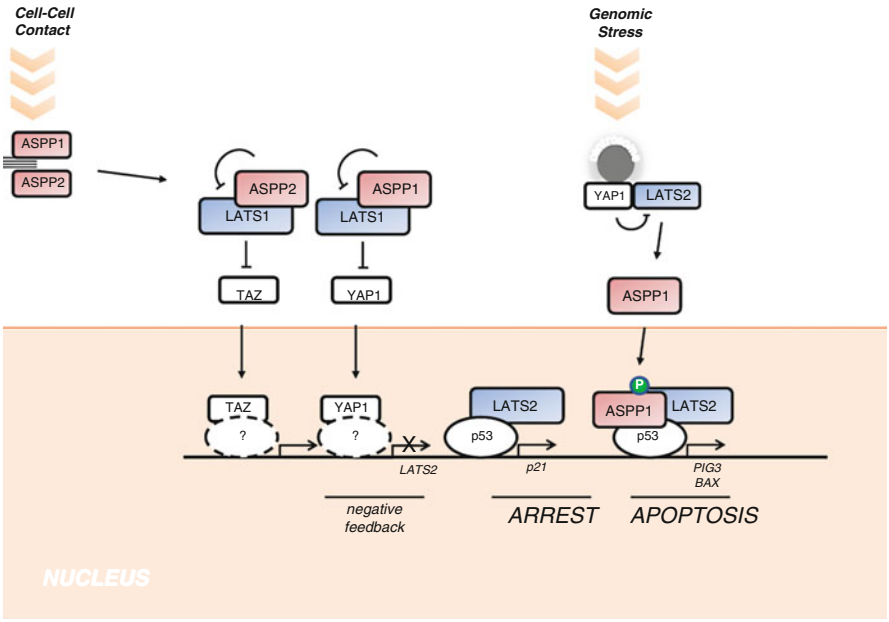

Fig. 7.4 The activity of LATS1/2 is differentially regulated by members id the ASSP family. The ASPP family of proteins which regulate p53 can differentially regulate LATS1/2 activity. Oncogenic H-Ras or nocodazole-mediated damage of the mitotic spindle promotes activation of LATS2 which phosphorylates ASPP1 and drives the nuclear translocation of LATS2 and ASPP1. The LATS2/ASPP1 complex binds p53 and promotes the selective activation of pro-apoptotic target genes and is required to prevent cells acquiring polyploid genomes. This interaction is blocked by YAP. In contrast, cells with high levels of cytoplasmic ASPP1 enhance YAP/TAZ activity by inhibiting the association of LATS1 with YAP, which prevents LATS1-dependent phosphorylation of YAP-Ser127 and nuclear accumulation of YAP. This promotes YAP-mediated repression of BIM, preventing the induction of apoptosis. YAP also negatively regulates the expression of LATS2 and indirectly inhibits p53. ASPP2 has also been shown to activate TAZ by promoting the dephosphorylation of TAZ and also preventing the binding of TAZ to the SCF E3 ubiquitin ligase which targets TAZ for proteasomal degradation

or post-translational modifications can control the switch between ASPP anti- and pro-apoptotic activities, and how in an untransformed cell a balance is achieved between ASPP-YAP-mediated growth and ASPP-LATS mediated cell death.

7.11.2 YAP, p73 and ΔNp63α

To date the best characterised pro-apoptotic effector of YAP is the p53 family member p73, which transcriptionally regulates many p53 pro-apoptotic target genes (Pietsch et al. 2008). The YAP-p73 complex has been shown to induce apoptosis in response to ionising radiation, chemically induced DNA damage, treatment with

FAS ligand or TNF and accumulation of the amyloid β peptide (Basu et al. 2003; Hamilton et al. 2009; Matallanas et al. 2007; Park et al. 2010; Strano et al. 2005; Yee et al. 2012; Zhang et al. 2011b). YAP binds to p63α and the p73α and β isoforms through a conserved WW domain and PPXY motif interaction and selectively enhances the recruitment of the YAP/p73 complex onto the promoters of pro-apoptotic genes in response to a range of DNA-damaging agents (Strano et al. 2001, 2005). Interestingly, neuronal cells expressing truncated variants of YAP lacking the C-terminal transactivation domain block p73-mediated cell death (Hoshino et al. 2006). Co-expression of YAP and p73 enhances the p73-dependent expression of pro-apoptotic genes and endogenous YAP-p73 are recruited to the promoters of *Bax*, *p53AIP1*, *Killer/DR5*, *PDCD5* and *PIG3* following treatment with DNA-damaging agents (Basu et al. 2003; Levy et al. 2008; Matallanas et al. 2007; Strano et al. 2001, 2005). Knockdown of YAP inhibits the expression of p73 target genes and subsequent cell death (Basu et al. 2003; Levy et al. 2008; Strano et al. 2005). There is a mutual requirement for YAP and p73 in the induction of apoptosis (Levy et al. 2008; Strano et al. 2005) as p73 is required for YAP translocation to the nucleus and YAP is necessary for p300-mediated acetylation of p73 which in turn promotes the induction of p73 pro-apoptotic target genes (Fig. 7.5a) (Costanzo et al. 2002; Strano et al. 2005). Additionally, post-translational modification of YAP also promotes the enrichment of the YAP-p73 transcriptional complex on the promoters of p73 pro-apoptotic genes. In response to DNA-damaging agents c-ABL phosphorylates YAP on Tyr375 (Levy et al. 2008). Phosphorylation of Tyr375 enhances YAP stability and promotes an increased association with p73. In addition, phosphor-Tyr375 is an important determinant in driving the selective activation of pro-apoptotic p73 target genes (Levy et al. 2008).

YAP is also required for the enhanced stability and accumulation of p73, and mutants of p73 that cannot bind YAP fail to be stabilised in response to DNA-damaging agents (Levy et al. 2007; Strano et al. 2005). YAP stabilises p73 by competing with the E3 ligase ITCH, which targets p73 for ubiquitination and proteasomal degradation. ITCH binds to p73 through the PPXY motif and YAP stabilises p73 by competing with ITCH for binding to this site (Levy et al. 2007). Furthermore, additional proteins have also been shown to be important in the promotion of YAP-p73-induced apoptosis. Promyelocytic leukaemia protein (PML) influences YAP-p73-induced apoptosis. PML is required for YAP-p73-mediated apoptosis and importantly as it is itself a YAP-p73 target, amplifies the induction of YAP-p73 target genes (Lapi et al. 2008; Strano et al. 2005). PML has several activities in this regard. It is required for YAP translocation to nuclear bodies (nuclear substructures with transcriptionally active regions of chromatin), and enhances p73 activity where it is detected in complex with YAP-p73 on the promoters of *Bax* and *p53AIP1* (Lapi et al. 2008; Strano et al. 2005) (Fig. 7.5). Moreover, PML enhances YAP stability, by promoting sumoylation of YAP on Lys97 and Lys242 and inhibiting YAP polyubiquitination and proteasomal-mediated degradation of YAP.

Although signalling through the core Hippo signalling cassette inhibits YAP in response to cell polarity, evidence suggests that under certain conditions, components of the core MST-LATS kinase cassette can induce apoptosis through the formation

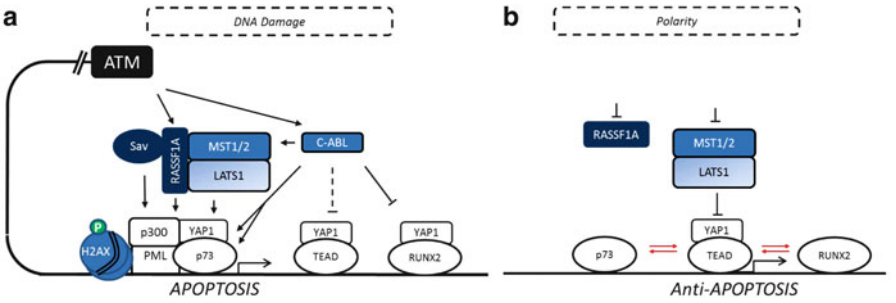

Fig. 7.5 The pro-apoptotic functions of YAP are promoted by RASSF1A. (**a**) RASSF1A is required for the formation of the YAP-p73 complex following DNA damage. Phosphorylation of RASSF1A via ATM promotes the MST2-LATS1-dependent formation of YAP-p73 and the expression of pro-apoptotic target genes. In addition, YAP-p73 is also subject to additional regulatory inputs through Tyr phosphorylation by c-Abl which promotes the selective activation of YAP-73 while inhibiting non-apoptotic YAP transcription factor complexes. MST1 also phosphorylates the H2A variant H2AX which is commonly phosphorylated by ATM following DNA damage. Thus MST1 and ATM may co-operate to amplify phosphorylation of H2AX which is required for the DNA damage response pathway. (**b**) The scaffold protein RASSF1A is an important determinant in regulating the transcription factor repertoire of YAP. Expression of YAP reduces the association of YAP and TEAD and if other binding partners of YAP such as RUNX2 are lost, RASSF1A further enhances the association of YAP with p73 and the inhibition of cell growth

and stabilisation of the YAP-p73 transcriptional complex (Fig. 7.5a) (Donninger et al. 2011; Hamilton et al. 2009; Kawahara et al. 2008; Matallanas et al. 2007; Park et al. 2010; Yee et al. 2012). In leukemic cells, loss of LATS2 expression prevents p73 stabilisation and the induction of target genes including p21 and BAX, resulting in resistance to chemotherapeutic drugs (Kawahara et al. 2008).

The tumour suppressor RASSF1A is a key determinant in driving the formation of the YAP-p73 complex via signalling through the MST1/2-LATS1/2 kinase cassette. RASSF1A promotes MST1/2 activation by releasing MST from the inhibitory RAF-1 complex and preventing its dephosphorylation (Guo et al. 2011; Matallanas et al. 2007; O'Neill et al. 2004; Romano et al. 2010). In response to DNA-damaging agents, RASSF1A promotes the YAP-p73-dependent expression of PUMA and BAX. (Hamilton et al. 2009; Matallanas et al. 2007; Yee et al. 2012). Indeed phosphorylation of RASSF1A Ser131 by ATM, a key kinase activated following DNA damage, is required for YAP-p73-induced gene expression in response to DNA damage (Hamilton et al. 2009). RASSF1A requires activation of MST2 and LATS1 for the expression of p73 target genes and siRNA-mediated knockdown of MST2; LATS1- or YAP blocks the expression of BAX and PUMA, inhibiting apoptosis. Interestingly RASSF1A also activates p73 in a manner that is partially independent of MST2 but dependent on SAV for p73 activity (Fig. 7.5a). However, RASSF1A requires MST2 for YAP nuclear localisation and stabilisation of p73 (Donninger et al. 2011). The molecular determinants of quite how RASSF1A promotes the formation of the YAP-p73 complex remain to be determined although new evidence

suggests that RASSF1A is an important determinant in defining the transcription factor repertoire of YAP (Fig. 7.5b) (van der Weyden et al. 2012). RASSF1A promotes YAP-p73 at the expense of other non-apoptotic YAP transcription factor complexes including YAP-TEAD and YAP-RUNX2. In this context it is likely that RASSF1A is promoting the interaction of YAP with additional regulatory proteins or altering the post-translational modifications of YAP which ultimately determine transcription factor preference (Fig. 7.5b). Indeed one such modification has been identified. The c-ABL phosphorylation of YAP-Tyr357 enhances the affinity of YAP for p73 while decreasing its interaction with RUNX1 (Levy et al. 2008). Thus post-translational modification is a clear regulator of YAP pro-apoptotic activity.

Recent reports suggest that the expression levels of the p63 isoform ΔNp63α may antagonise YAP-p73-mediated apoptosis as ΔNp63α has been shown to inhibit p73-mediated apoptosis (Rocco et al. 2006). YAP interacts with p63 and ΔNp63α and is required for ΔNp63α-mediated resistance to apoptosis in response to cisplatin (Strano et al. 2001; Yuan et al. 2010a). In response to cisplatin, c-ABL phosphorylation of both YAP (Tyr357) and ΔNp63α (Tyr 55, Tyr137 and Tyr308) promotes the formation of the YAP/ΔNp63α complex which inhibits the induction of apoptosis (Yuan et al. 2010a). Furthermore, in response to UV irradiation, JNK-mediated phosphorylation of YAP (Ser 138, Ser317,Thr362) promotes the stabilisation of ΔNp63α, by competing with ITCH for binding to ΔNp63α in an analogous manner to YAP-mediated stabilisation of p73 (Levy et al. 2008; Tomlinson et al. 2010). Indeed, it has been suggested that the elevated expression of ΔNp63α may protect against YAP-p73-driven apoptosis (Tomlinson et al. 2010).

It is of interest to note that p63 contains the same residues that are phosphorylated by JNK and c-ABL. c-ABL-dependent phosphorylation of p63 induces the expression of NOXA and PUMA which induce apoptosis (Gonfloni et al. 2009). It will therefore be of interest to determine the role of YAP in p63-mediated apoptosis.

7.12 Summary

It is now clear that the Hippo pathway has an important, evolutionary conserved role in the regulation of the apoptotic response. Many parallels can be drawn between the pathway in *Drosophila* and mammals. The anti-apoptotic transcriptional programme of Yki and YAP shares common targets, with transcription of members of the IAP family promoting the protection of cells from apoptosis. The current understanding of Hpo and Wts in driving apoptosis in *Drosophila* suggests that phosphorylation-dependent inactivation of Yki and inhibition of Diap-1 and Bantam transcription are key to the induction of developmental apoptosis. Multiple regulators of Bantam, both ubiquitous and tissue specific, have been characterised and it is likely that other yet unidentified Yki transcriptional complexes are responsible for the regulation of Diap-1 transcription.

In the mammalian system the MST and LATS kinases function in multiple pro-apoptotic pathways and can regulate the activity of multiple transcription factors that are classically associated with apoptosis, resulting in the induced expression of pro-apoptotic members of the bcl-2 family. In addition, MST1/2 also promote histone-mediated chromatin condensation via H2B and H2AX phosphorylation, allowing controlled destruction of the nucleus through fragmentation of genomic DNA. Some open questions remain regarding the upstream activators of both MST and LATS and although we know that caspases and RASSF family members promote MST1/2 there are likely to be other routes to activate this kinase and importantly we do not know if apoptotic pathways regulated by LATS1/2 are dependent on MST. In the mammalian system, YAP can both inhibit and promote apoptosis and the MST-LATS kinase cassette is required for both these activities. Although we are now beginning to understand some of the players such as RASSF1A and the expression of ΔNp63α that differentiate YAP pro- or anti-apoptotic activity, the molecular details of what determines the switch in the choice of YAP in transcription factor binding remain unknown. It is now clear that in cancer, inhibition of apoptosis is a hallmark that drives tumour growth. Indeed inactivation of MST (Matallanas et al. 2011) or LATS (Aylon et al. 2009) has been proposed as a mechanism of bypassing apoptosis. However, the most common mechanism in inhibiting Hippo pathway-mediated apoptosis is through the methylation of the RASSF family proteins which are a frequent occurrence in cancer (Richter et al. 2009). Not only does loss of RASSF1A reduce the activation of MST, it also impacts on the pro-apoptotic activities of YAP. Moreover, failure to initiate apoptosis alone may lead to enhanced proliferation through promotion of YAP proliferative complexes; however recent evidence suggests that sustained proliferation of human tumours frequently requires concomitant loss of YAP differentiation cofactors (Van der weyden et al. 2012).

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Chapter 8
YAP and p73: A Matter of Mutual Specificity in Tumor Suppression

Sara Donzelli, Sabrina Strano, and Giovanni Blandino

Abstract YAP and p73 proteins are key nodes of two distinct tumor suppressor pathways. The HIPPO tumor suppressor pathway to which YAP belongs is the most recent identified in the cancer arena, while that of the p53 family including p73 is the most well studied and characterized. Often in response to anticancer treatment, distinct tumor suppressor pathways can be triggered and cross talk each other. This is well represented by the growing experimental evidence linking HIPPO and p53 family tumor suppressor pathways. Here we mainly focus on the physical and functional interaction between YAP and p73 proteins, their role in drug-induced apoptosis and their implications in tumor suppression.

Keywords p73 • YAP • Apoptosis • Interaction • Pathway

8.1 The p73 Gene

The p73 gene, which is extensively subjected to alternative splicing, encodes proteins that are almost equally distributed among TA and ΔN isoforms. The resulting proteins are members of the p53 family. They are heavily involved in growth suppression, apoptosis, DNA repair, senescence, and differentiation.

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_8,

8.1.1 p53 Family

The first member of the family identified was p53 in 1979, whose tumor suppressor activity is now well established (Lane and Crawford 1979; Linzer and Levine 1979). It is in fact defined "the guardian of the genome," due to its ability to induce cell cycle arrest or apoptosis in the presence of different kinds of cellular stress signals. It is also found mutated in almost 50 % of human cancers (Hainaut et al. 1997; Hollstein et al. 1997).

The other two members of the family, p63 and p73, were discovered in 1997, almost 20 years after discovering p53. Even if they were discovered later than p53, the overall structure and sequence homology suggest that a p63/p73-like protogene is the ancestral gene, whereas p53 evolved later in higher organisms.

p63 and p73 are structurally and functionally similar to p53 sharing the same function. For instance their involvement in tumor suppression (Collavin et al. 2010). However, they also have specific functions that differ from p53, that is, playing important roles in embryonic development and differentiation (Jost et al. 1997; Kaghad et al. 1997; Yang et al. 1998).

The p53 transcription factor family members are characterized by a very similar protein structure composed of an amino-terminal transactivation domain (TA), a proline-rich domain (PR), a core DNA binding domain (DBD), and a carboxy-terminal oligomerization domain (OD) (Blandino and Dobbelstein 2004; Levrero et al. 2000; Melino et al. 2002; Stiewe and Putzer 2002). In contrast to p53, p63 and p73 proteins contain also a sterile-alpha motif (SAM) domain at the carboxy-terminal region. Knowledge of these domains' specific functions is still poorly understood. It seems to mediate the interaction with other proteins not yet identified (Chi et al. 1999). The SAM domain is made up of typical proteins that take part in development regulation, thus supporting the involvement of p63 and p73 in differentiation (Harms and Chen 2005; Irwin and Kaelin 2001; Ozaki et al. 1999).

The DBD is the domain with the highest grade of homology between the three family members, with 65 % of homology between p53 and p73. This results in the activation of overlapping sets of target genes.

Conversely, the OD domain is less conserved among the family members; p53 binds to DNA as a homo-tetramer. While p63 and p73, preferentially form homo-tetramers rather than hetero-tetramers with each other (Davison et al. 1999; Marin and Kaelin 2000).

The gene structure of p53 family members is highly complex and gives rise to different protein isoforms (Mills 2005; Murray-Zmijewski et al. 2006). Among the p53 family members, the p73 gene is the most complex and presents the highest number of isoforms generated by the activity of two distinct promoters on p73 gene and by different alternative splicing events. The resulting proteins may have antagonistic properties and are expressed differently in normal human tissue and cell lines.

8.1.2 p73 Protein Isoforms

The p73 gene is composed of 15 exons spanning over 80,000 bp on chromosome 1p36.32 (Ozaki and Nakagawara 2005). The biology of p73 gene is highly complex, since it can be transcribed in a variety of different isoforms generated by alternative splicing events and by the activity of two distinct promoters, for a total of 45 mRNA variants. These can encode theoretically 36 different p73 protein isoforms (Fig. 8.1).

Alternative splicing events between exons 10–13 at the C-terminal give rise to seven different isoforms (p73 α–η) (De Laurenzi et al. 1998; Kaghad et al. 1997; Melino et al. 2002; Moll and Slade 2004). Among these, p73α is the longest one containing a SAM in the extreme C-terminal region. C-terminal splicing isoforms display different transcriptional and biological properties (De Laurenzi et al. 1998; Ozaki et al. 1999; Ueda et al. 1999). Indeed, p73β has a stronger effect in the transactivation of p53/p73 target genes and in the induction of apoptosis in cancer cells than the full-length p73α (Lee and La Thangue 1999; Ueda et al. 1999). This suggests the existence of a regulatory function for the C-terminal domain (Lee and La Thangue 1999; Ozaki et al. 1999). Moreover, different splicing isoforms are differentially expressed among human tissues and cell lines, thus presenting different biological functions (De Laurenzi et al. 1998; Ueda et al. 1999).

In addition to C-terminal isoforms, alternative splicing events on the p73 gene give rise also to four N-terminal variants initiated at different ATG (ΔN′ p73, Δ2p73, Δ3p73, Δ2,3p73) (Fillippovich et al. 2001; Ishimoto et al. 2002; Murray-Zmijewski et al. 2006). These truncated forms, named ΔNp73 are transactivation-defective and behave as dominant negative isoforms in regard to TAp73 and p53 and act as anti-apoptotic proteins (Pozniak et al. 2000).

The other isoforms of p73 are generated by the activity of two distinct promoters present on the p73 gene: P1, located immediately upstream from the first exon, and P2, located in intron 3 upstream from the transcription starting site for ΔNp73 within exon 3′. The P1 and P2 promoters give rise to full-length TAp73 isoforms, and amino-terminal truncated ΔNp73 isoforms, respectively. The transcripts are exposed to both amino- and carboxy-terminal splicing.

The TAD is required for interacting with different transcription coactivators that allow the enhanced expression of p53 target genes. As a result, the TAp73 isoforms are able to induce the expression of different p53-responsive genes, such as p21, GADD45, PUMA, and BAX, controlling growth arrest and apotosis. Conversely, the ΔNp73 isoforms act as dominant negative proteins with anti-apoptotic and pro-proliferative effects by inhibiting TAp73 and p53: the ΔN variants can occupy p53-responsive promoters by preventing on them the transcription machinery recruitment or can interact with TAp73 isoforms generating inactive hetero-tetramers (Pozniak et al. 2000; Yang et al. 2000).

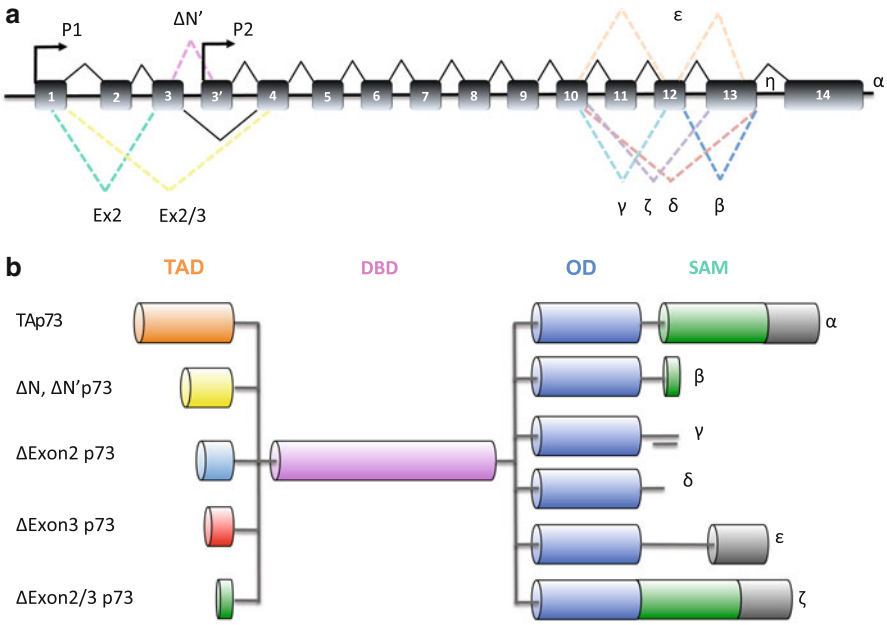

Fig. 8.1 Structure of p73 gene and its isoforms. (**a**) Structure of the human p73 gene. The p73 gene is located on chromosome 1p36.32 and is composed of 15 exons. The transcripts are generated by two alternative promoters (P1 and P2) and undergo alternative splicing events that give rise to different isoforms. (**b**) p73 isoforms. Similar to the other p53 family member, p73 protein is composed of an amino-terminal transactivation domain (TA), a core DNA binding domain (DBD), a carboxy-terminal oligomerization domain (OD), and a sterile-alpha motif (SAM) that is present only in p73 and p63. TA variants are generated by the P1 promoter and possess a functional TAD, while ΔN isoforms, generated by the P2 promoter, lack the TAD domain and thus are transcriptionally inactive. ΔN isoforms can also be generated by alternative splicing events at the first two exons. All the transcripts can also be subjected to alternative splicing events at the C-terminal exons, thus generating a variety of isoforms

8.1.3 p73 Functions

Unlike the p53 gene product that is ubiquitously expressed, the p73 protein shows tissue specificity. In addition, it is involved in defining the developmental stage.

The role of p73 in neurogenesis, sensory pathways, and homeostatic control is revealed by $p73^{-/-}$ mice that present congenital hydrocephalus, hippocampal dysgenesis, loss of peripheral-sympathetic neurons, chronic infections and inflammations, and defects in pheromone detection (Pozniak et al. 2000; Yang et al. 2000).

Differently from $p53^{-/-}$ mice, p73 knockout mice do not show increased susceptibility to spontaneous tumorigenesis, since they die 4–6 weeks after birth (Yang et al. 1999, 2000).

Many of the neurological defects observed in the $p73^{-/-}$ mice are a consequence of neuron absence or loss (Yang et al. 2000). During the development of the nervous system, neurons are overproduced and cells directly compete in order to survive or die, for their growth factors in a process known as naturally occurring cell death. The neurons that are usually committed to surviving during the developmental apoptosis instead die in the p73 knockout mice (Pozniak et al. 2000). In the $p73^{-/-}$ mice, the absence of ΔNp73, the isoform more expressed in murine fetal nervous system, could be responsible for an enhanced neuronal apoptosis (Pozniak et al. 2000, 2002).

Like its homologue p53, p73 has also been implicated in cellular senescence (Alexander et al. 2003; Fang et al. 1999). In p53-depleted tumor cells, the overexpression of p73α and p73β isoforms is sufficient to induce permanent growth arrest with markers of replicative senescence (Fang et al. 1999).

8.1.4 p73 and Apoptosis

A range of chemotherapeutic drugs induce apoptosis via signaling pathways where p53 and/or p73 are central players (Erster and Moll 2005). Chemotherapeutic drugs promote p53 and/or p73 stabilization and activation, which in turn exert transcription-dependent and -independent effects leading to cell cycle arrest or apoptosis. It has been shown that the inhibition of p73 functions reduce the cytotoxicity of chemotherapeutic agents, hence underlying the important role of p73 in controlling cellular sensitivity to some anticancer treatments (Melino 2003; Sayan et al. 2008).

p73 plays an important role in apoptosis as it is involved in the activation of both intrinsic (mitochondria-mediated), extrinsic (death receptor-mediated), and endoplasmic reticulum-mediated apoptotic pathways.

The majority of proteins involved in p73-mediated apoptosis are controlled at the transcriptional level by p73, that is the case of Bax (Di Como et al. 1999; Zhu et al. 1998), PUMA (Melino et al. 2004), Noxa (Wang et al. 2008), CD95 (Muller et al. 2005), and Scotin (Terrinoni et al. 2004).

The activation of Bax, PUMA, and Noxa creates a link between p73 and the mitochondria-mediated cell death pathway (Flinterman et al. 2005; Ramadan et al. 2005). The transcriptional upregulation of CD95 induce the receptor-mediated cell death pathway (Muller et al. 2005), while the induction of Scotin promotes cell death by the induction of endoplasmic reticulum-stress and changes in intracellular calcium levels (Terrinoni et al. 2004).

The p73 exerts also a transcription-independent pro-apoptotic activity: upon death-receptor activation p73 is cleaved by caspases; the p73 fragment and also the full length p73 translocate to the mitochondria where they promote cytochrome c release (Sayan et al. 2008).

As mentioned above, ΔNp73 isoform has a complete opposite effect compared to TA isoform: it prevents apoptosis by different mechanisms. This effect is due principally to its dominant negative effect on TAp73 and p53 transcriptional activity (by competing with TAp73 and p53 for the binding sites on the promoters of their target genes and by the formation of inactive complexes with TAp73 isoforms).

A result of this effect is the ΔNp73 inhibition of Bax, CD95, and Scotin transcriptional activation (Muller et al. 2005; Rossi et al. 2004). The anti-apoptotic activity of ΔNp73 isoform is also exerted by the activation of the heat shock factor (HSF)-responsive gene expression, such as the anti-apoptotic inducible heat shock protein (Hsp)-72 (Tanaka et al. 2004).

8.1.5 p73 in Cancer

Despite the significant homology to p53, the p73 gene is not a classic Knudson-type tumor suppressor gene (Melino et al. 2002).

The first gene targeting studies in the mouse indicated that p73 was mainly implicated in normal development (Yang et al. 2000). It did not seem to be involved in cancer as $p73^{-/-}$ mice did not display an increased susceptibility to spontaneous tumorigenesis. However, this effect was due to the fact that the construct utilized to generate $p73^{-/-}$ mice deleted the central DBD of p73, so it affected all the p73 isoforms. In addition, the phenotype observed was the result of both TA and ΔN variant depletion, that, as mentioned above, have an opposite biological effect. In fact there are no changes in the balance of TA/ΔN isoforms, and also in their pro-apoptotic and anti-apoptotic effects, respectively.

The role of p73 in cancer prevention was clearly highlighted by the generation of p73 heterozygous mice alone or in combination with p53. It has been shown that $p73^{+/-}$ and $p73^{+/-}$:$p53^{+/-}$ mice present more aggressive tumor phenotypes compared to $p73^{+/+}$ and $p73^{+/+}$:$p53^{+/-}$ mice (Flores et al. 2005).

Moreover, the generation of specific TAp73 knockout mice ($TAp73^{-/-}$), in which the expression of the ΔN isoforms is still maintained, confirmed TAp73 tumor suppression activity. Compared to $p73^{-/-}$ mice, the $TAp73^{-/-}$ mice present less hippocampal dysgenesis, but increased infertility due to genomic instability of the oocyte and demonstrating a very high incidence of spontaneous tumors, particularly lung adenocarcinomas (Tomasini et al. 2009). The $TAp73^{-/-}$ mice phenotype indirectly shows the oncogenic potential carried by ΔNp73 isoforms, highlighting the importance of a proper balance between TA and ΔN isoforms in normal cells in maintaining genomic fidelity.

In contrast to p53 that is mutated in 50 % of human cancers, the p73 gene is rarely mutated having a frequency of 0.5 % (Ikawa et al. 1999; Nimura et al. 1998; Nomoto et al. 1998; Shishikura et al. 1999; Takahashi et al. 1998). Two examples of p73 mutation are P405R and P425L, a somatic and germ line mutation found in primary neuroblastomas (Naka et al. 2001).

Even if the p73 gene is rarely mutated, it shows a significant incidence in the loss of heterozygosity in a number of different tumors.

Moreover, the p73 gene is often upregulated in different cancer types and this is associated with a poor patient survival prognosis (Tannapfel et al. 1999).

With the design of reagents directed toward discriminating different p73 splicing variants, it was possible to verify that the ΔNp73 isoform is the predominately over-expressed one in tumors of the lung, breast, thymus, colon, prostate, skin, ovary, muscle, and other organs, whereas it is not expressed in healthy tissues (Bozzetti et al. 2007; Cam et al. 2006; Casciano et al. 2002; Dominguez et al. 2006a, b; Frasca et al. 2003; Guan and Chen 2005; Uramoto et al. 2004, 2006; Wager et al. 2006).

In vitro and in vivo studies confirmed the oncogenic role of ΔNp73 isoform. It promotes fibroblasts colony formation ability (Ishimoto et al. 2002) and cooperates with k-RAS, c-myc, and E1A in promoting transformation and tumorigenicity (Petrenko et al. 2003).

Other evidence of ΔNp73 oncogenic role is significantly shown from transgenic mice in which ΔNp73 was overexpressed in the liver. The mice displayed an increased proliferation of hepatocytes, with acinar disorganization and the appearance of pre-neoplastic nodules that in the 83 % of cases evolved in hepatic carcinoma (Tannapfel et al. 2008).

However in various studies, like ΔNp73, also TAp73 expression levels were found increased. This greater increase in levels is consistent with those studies demonstrating the ability of TAp73 in transactivating the ΔNp73 promoter and that ΔNp73 in turn stabilizes the TAp73 protein (Becker et al. 2006; Bozzetti et al. 2007; Dominguez et al. 2006a, b; Frasca et al. 2003; Grob et al. 2001; Slade et al. 2004). In these cases, the tumor suppressive activity of TAp73 is counteracted by a dominant negative effect of ΔNp73 that is able to occupy the DNA binding sites on the promoters of p53 and p73 target genes and to form inactive hetero-tetramer with TAp73 (Pozniak et al. 2000).

8.1.6 p73 Regulation

The expression and the activity of p73 isoforms can be modulated at three different levels (Fig. 8.2): (a) transcriptional regulation of P73 gene P1 and P2 promoters that determines which amino-terminal isoform is produced; (b) post-transcriptional regulation of gene expression by the use of alternative carboxy- and amino-terminal splicing; (c) post-translational regulation that has an impact on protein stability, on protein–protein interaction, and on specificity to target genes (Marabese et al. 2007).

(a) As mentioned above, there are two distinct promoters present on the p73 gene: P1 located upstream from the exon 1 and P2 located within intron 3 which give rise to TA and ΔN isoforms, respectively.

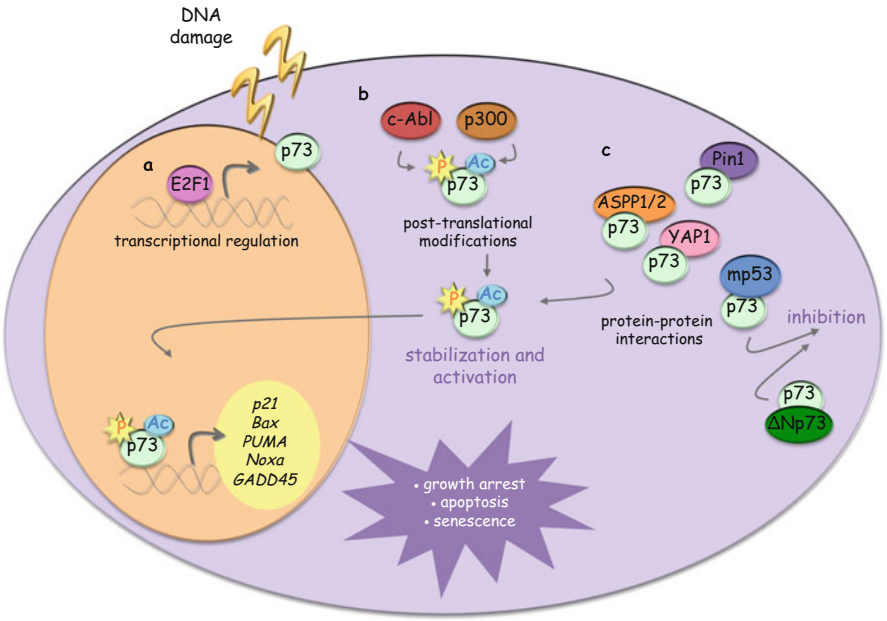

Fig. 8.2 p73 regulation. p73 activity is regulated at different levels: (**a**) transcriptional regulation on P1 and P2 promoters by different transcription factors among which E2F1 on P1 promoter is the best characterized. Upon DNA damage E2F1 binds to its specific consensus sequences on P1 promoter inducing TA isoforms transcription; (**b**) post-translational regulation by protein modifications such as phosphorylation, principally by c-Abl, and acetylation by p300 that stabilize and therefore activate p73; (**c**) physical interaction with other proteins that promote p73 activation (such as ASPP1/2 and YAP) or its inhibition (such as mutant p53 or ΔNp73). Subsequently to its activation, p73 binds to its target genes and, depending on the type of stimulus, it promotes cell growth arrest, apoptosis, or senescence

Binding sites are present on the P1 promoter for different transcription factors such as E2F, Sp1, Myc, c-Myb, AP-2, Egr-1/-2/-3, NFAT, and homeobox proteins (De Laurenzi and Melino 2000). There are also several stretches of CpG islands upstream from the P1 promoter (Ding et al. 1999).

Among the transcriptional regulators of p73, the best characterized one is E2F1. It controls p73 expression under physiological conditions, such as in the G1/S transition or in activation-induced cell death of thymocytes (Irwin et al. 2000; Lissy et al. 2000; Stiewe and Putzer 2000). It has been demonstrated that upon DNA damage E2F1 is subjected to post-translational modifications, such as phosphorylation and acetylation. In addition, it is selectively recruited on the P1 promoter to activate it and to promote a cellular apoptotic response (Ozaki et al. 2009; Pediconi et al. 2003; Urist et al. 2004). E2F1 deacetylation by Sirt1 leads to inhibition of TAp73 transcription (Pediconi et al. 2009). Also methylation and demethylation of E2F1 by Set9 and LSD1 enzymes, respectively, is critical for E2F1 activity on P1 promoter (Kontaki and Talianidis 2010).

E2F1 activity on P1 promoter is also regulated by the interaction with other factors that act as transcriptional repressors, such as C-EBPα, or transcriptional activators, such as YY1 (Marabese et al. 2003; Wu et al. 2008).

In addition to E2F1, cellular and viral oncogene products such as c-Myc and E1A indirectly activated the transcription of p73 (Urist et al. 2004).

A 1-kb regulatory region within the first intron of p73 has also been identified, immediately upstream from the ATG codon of exon 2, containing six consensus binding sites for the transcriptional repressor ZEB1 (Fontemaggi et al. 2001).

Interestingly, the P2 promoter does not contain an E2F1 binding site but possesses a highly efficient p53/TAp73-responsive element. This promoter is activated in response to non-apoptotic DNA damage in a p53-dependent manner to accumulate ΔNp73 proteins and to determine p53/p73-induced cell cycle arrest in cells that do not undergo apoptosis (Grob et al. 2001; Kartasheva et al. 2002; Nakagawa et al. 2002; Vossio et al. 2002). The control of p53/TAp73 on ΔNp73 promoter creates a negative autoregulatory feedback loop that can finely regulate p53 family functions. The loss of these negative feedback loops, occurring in cancer or in infected cells, would result in an indefinite increase in ΔNp73 expression that consequently inactivates p53 and p73, contributing to cancer development (Allart et al. 2002; Stiewe et al. 2002).

(b) The alternative splicing events that occur on the human p73 gene can theoretically give rise to 45 mRNA transcripts which in turn could potentially encode 36 different proteins. The different isoforms are differentially expressed in the human tissues and present specific functions.

(c) Post-translational regulation of p73 includes protein modifications, such as phosphorylation and acetylation and physical interactions with other proteins. These events have an important effect on p73 protein stability and specificity to target genes.

Upon DNA damage, p73 protein is stabilized and thereby activated by the activity of different protein kinases: c-Abl phosphorylates p73 at tyrosine residue 99 (Yuan et al. 1999); Chk1 phosphorylates p73 at serine residue 47 (Gonzalez et al. 2003); PKCδ phosphorylates p73 at serine residue 289 (Ren et al. 2002). All these modifications promote p73 apoptotic activity.

c-Abl also induces p73 phosphorylation in threonine residues adjacent to prolines and the p38 MAP kinase pathway mediates this response (Sanchez-Prieto et al. 2002). Furthermore, p38 phosphorylation of p73 is required for p73 stabilization and recruitment into the nuclear bodies.

The promyelocytic leukemia (PML) protein, in fact, modulates p73 half-life by inhibiting its ubiquitin-proteasome degradation in a PML-nuclear body-dependent manner. PML regulates p73 stability by positively modulating the levels of acetylation, a process that impairs p73 ubiquitination.

As a result, PML potentiates p73 transcriptional and pro-apoptotic activities that are markedly impaired in $PML^{-/-}$ primary cells (Bernassola et al. 2004).

The acetylation of specific p73 protein residues has a pro-apoptotic function effect. Upon doxorubicin treatment p300 acetylates p73 at lysine residues 321, 327 and 331 in a c-Abl-dependent manner (Costanzo et al. 2002). The physical interaction

of p73 with the prolyl isomerase Pin1 promotes p300-mediated acetylation by inducing conformational changes on p73 (Zeng et al. 2000).

Another important post-translational modification that controls p73 stability is the sumolation. Like p53, p73 interacts with the small ubiquitin-like modifier (SUMO) protein which in turn covalently modifies p73α, but not β, on the C-terminal lysine 627 residue and promotes p73α proteasomal degradation (Minty et al. 2000; Rodriguez et al. 1999).

p73 can also be differentially regulated by ubiquitination. NEDL2 ubiquitination within C-terminal proline-rich motif enhances p73 transcriptional and pro-apoptotic activity (Miyazaki et al. 2003). Alternatively, ubiquitination by the ubiquitin E3 ligase Itch potentiates p73 proteasomal degradation. DNA damage causes down regulation of Itch through a poorly characterized mechanism, thus allowing the stabilization and activation of p73 (Agami et al. 1999; Gong et al. 1999; Rossi et al. 2005; Yuan et al. 1999).

The activity of p73 is also influenced by the physical interaction of several proteins. An example of positive regulation is the interaction with ASPP1 and ASPP2. Their binding to p73 DBD specifically stimulates the transactivation functions of p73 on the promoters of pro-apoptotic genes, such as Bax and Puma, except on the promoter of p21 and Mdm2 (Bergamaschi et al. 2004). The ASPP inhibitor family member (iASPP) counteracts this triggering effect on p73 (Bergamaschi et al. 2003; Robinson et al. 2008; Samuels-Lev et al. 2001; Vermeulen et al. 2003).

Also the interaction with the p73 target gene products, Cyclin G and Mdm2, has a negative effect on p73 activity. Cyclin G binding to p73 promotes its degradation, while Mdm2 binding to the transcriptional coactivator p300 causes the disruption of physical and functional interactions between p73 and p300 (Ohtsuka et al. 2003; Zeng et al. 1999).

Of particular interest is the interaction between mutant p53 and p73. The p53 gene is known to be mutated in 50 % of human cancers. The most prevalent types of p53 alterations are missense mutations that occur in the DBD. These mutations result in a very significant loss of DNA binding activity and transactivation capacity (Ory et al. 1994). It is increasingly evident that many mutant p53 forms not only lose their tumor suppressive functions and acquire dominant negative activities, but also gain new oncogenic properties that are independent of wild-type p53 and actively sustain tumor development and progression (Brosh and Rotter 2009). One of the mechanisms by which mutant p53 exerts its gain of function activity is the ability to bind through its mutated DBD and to inhibit its family members, p73 and p63, blocking the transactivation of downstream target genes involved in apoptosis and growth arrest control (Adorno et al. 2009; Di Como et al. 1999; Gaiddon et al. 2001; Marin and Kaelin 2000; Strano and Blandino 2003; Strano et al. 2000). In this regard, some short synthetic peptides, capable to physical disrupt the mutant p53/p73 interaction, have been recently engineered; their effect is to sensitize mutant p53 tumor cells to cisplatin and adryamicin treatments (Di Agostino et al. 2008).

Also the p53 polymorphism in codon 72 (Arg or Pro) influences the interaction with p73, in particular the Arg in the 72 position is required to bind p73 (Marin et al. 2000).

Finally another interactor of p73 that has an important impact on its stability and its activity is the transcriptional coactivator Yes-associated protein 1 (YAP1), a key node of the Hippo signaling pathway. This physical interaction occurs between the PPPPY motif of p73 and the WW domain of YAP and it is specific for only the p53 family members that have a well-conserved PPXY motifs (not p53). The result of YAP protein binding is an enhancement of p73 transcriptional activity upon DNA damage and increase in p73 stability, since YAP promotes the E3 ubiquitin ligase Itch dissociation from p73 (Basu et al. 2003; Strano et al. 2001, 2005). The importance of these interactions will be discussed below.

8.2 YAP

Discovered in 1994 as a 65 kDa binding partner of the Src family kinase c-Yes, chicken YAP (Yes-Associated Protein) was named YAP65 (Sudol 1994). Mouse and human homologs were subsequently cloned and characterized the next year (Sudol et al. 1995).

Two different isoforms have been identified: YAP1 and YAP2, where the principal difference consists in the presence of 1 or 2 WW domains, respectively. Regulation of the switch between the two YAP isoforms is not clear. YAP2 is the major isoform in humans (Komuro et al. 2003).

In general, YAP mRNA is ubiquitously expressed in a wide range of tissues, except peripheral blood leukocytes (Komuro et al. 2003). YAP is also expressed in the full developmental stages from blastocyst to perinatal (Morin-Kensicki et al. 2006).

8.2.1 YAP Functions Discovery

YAP protein was first characterized as a transcriptional coactivator able to bind the PPxY motif of Runx1 (or AML1 for acute myeloid leukemia 1) (Yagi et al. 1999).

Then it was reported that YAP, like its paralog TAZ, presents a 14-3-3 binding site. Affinity purification of the epitope-tagged TEAD2/TEF4 (TEA domain protein 2/transcriptional enhancer factor 4) revealed that YAP is in stable complex with 14-3-3 proteins. In addition, it showed that these proteins seemed to regulate the role of YAP as a coactivator of the TEAD/TEF family of transcription factors (Vassilev et al. 2001). According to these data it was proposed that YAP and its paralog TAZ are 14-3-3 binding transcriptional coactivators regulated by dynamic nucleo-cytoplasmic trafficking. 14-3-3 proteins are phosphoserine- and phosphothreonine-binding signaling regulators for CDC25 phosphates, transcription factors, histone deacetylase, and many others (Yaffe and Elia 2001).

Hence, an important question to pose is which kinases are responsible for phosphorylation of YAP and for linking its regulation to cellular signaling networks? Much support in studying YAP functions came with the identification of Yorkie, the fly ortholog of YAP, that was discovered in a yeast 2-hybrid screening aimed at identifying partners of *Drosophila* M. kinase Warts/Lats (large tumor suppressor) (Huang et al. 2005). This kinase phosphorylates Yorkie resulting in the inhibition of its transactivating and tissue growth-promoting activities (Huang et al. 2005). The kinase Warts/Lats functions downstream from the Hippo kinase (an Ste20 family member) as a key component of a novel signaling pathway important for organ size control (Pan 2007). Thus, the unexpected Yorkie-Lats link suggests the regulation of YAP (and TAZ) by the Hippo-like pathways in mammals. Indeed, several groups have recently shown that mammalians Lats and Hippo-like kinases control the subcellular localization, transcriptional coactivator activities, and biological function of YAP (Camargo et al. 2007; Dong et al. 2007; Oka et al. 2008; Zhang et al. 2008).

Recently, Dupont et al., have identified YAP and its paralog TAZ as nuclear translators of mechanical signals exerted by extracellular matrix stiffness and cell shape. In particular, YAP and TAZ are required for the mesenchymal stem cells differentiation induced by extracellular matrix rigidity and for endothelial cell survival regulated by cell geometry (Dupont et al. 2011).

8.2.2 WW Domain Functions

The WW domain is able to bind short stretches of prolines or PY motifs, therefore mediating the interaction between proteins. The WW domain of YAP belongs to the first of four different classes that differ in terms of sequence of the interacting motif, a PPxY motif in the case of WW type I. The WW domain appears to contain β-strands grouped around four conserved aromatic positions (Bork and Sudol 1994). Other important features of WW domain are the large amount of polar amino acids and the presence of prolines distributed preferentially toward both termini of the linear sequence. One of the C-terminal prolines seems strictly invariant. The length of the domain is of approximately 38 residues as suggested by the length of the second WW module identified in the mouse ortholog of YAP (Sudol et al. 1995).

YAP has been found to interact with many proteins whose functions are often different between them and the majority of these interactions mostly occur through WW domain. For example, YAP is able to bind the cytosolic tail of the Erb4 receptor. Upon binding to its ligand, this receptor undergoes cleavage by intra-membrane proteases that free the cytosolic portion. Despite the fact that this interaction occurs in the cytoplasm, YAP is needed to regulate the activity of the cleaved part of Erb4 only once it gets into the nucleus (Komuro et al. 2003; Omerovic et al. 2004).

In addition YAP1 was also found to partner Smad7, enhancing the inhibitory activity of Smad7 against the TGFbeta/Smad1,-2,-3,-5 signaling complex (Ferrigno et al. 2002). The interaction with PEBP2 (an RUNX transcription factor) was the first example of YAP1 as a coactivator of transcription. The WW domain of YAP1

interacts with the PY motif present in the transcription activation domain of PEBP2. In this occasion, YAP1 was reported for the first time to have strong intrinsic transactivation activity (Yagi et al. 1999).

8.2.3 YAP Activity Regulation

The principal mechanism through which YAP protein activity is regulated consists in the control of its cellular localization: YAP exerts its activity into the nucleus and when it is retained into the cytoplasm it is inactive. The nucleo-cytoplasmic shuttling of YAP is dependent on post-translational modifications of the protein, mainly phosphorylations, that promote YAP binding and therefore sequestering by 14-3-3 protein. The residue mainly undergoing phosphorylation is the serine 127 and the first kinase identified as responsible for this modification was Akt/PKB (Basu et al. 2003). It was proposed that in response to DNA damage Akt/PKB is activated. It induces YAP phosphorylation and subsequently retention into the cytoplasm. Therefore suppressing its ability to induce apoptosis through the transcriptional activity of p73 (Basu et al. 2003; Strano et al. 2005).

Other kinases demonstrated to be able to phosphorylate serine 127 residue of YAP are LATS1 and LATS2 (Hao et al. 2008; Oka et al. 2008; Zhao et al. 2007).

As previously mentioned, YAP was firstly discovered as a protein interacting with the two tyrosine kinases c-Yes and c-Src, so it is reasonable to expect post-translational modifications of YAP by these two proteins. In fact, the ability of c-Src/Yes to bind to YAP and to phosphorylate its tyrosine residues promoting YAP recruitment of RUNX2 to subnuclear sites was demonstrated, which in turn results in RUNX2 activity inhibition (Zaidi et al. 2004).

Also the kinase c-Abl is involved in YAP activity regulation. Upon DNA damage, c-Abl phosphorylates the tyrosine 357 residues of YAP promoting its stability and its affinity for p73 (Levy et al. 2008). As mentioned above, c-Abl has an effect also on p73: c-Abl phosphorylation of p73 tyrosine 99 residue promotes its apoptotic activity (Agami et al. 1999).

8.2.4 YAP Protein Structure

YAP protein is composed of several distinct domains (Fig. 8.3): a proline-rich region at the N-terminal, important for the interaction with the SH3 domain of many proteins, among which is c-Yes; two tandem WW domains in the middle, the name originates from the presence of two tryptophan residues, which appear to be conserved along evolution and play an important role in the domain structure and function (Huang et al. 2000; Sudol et al. 1995; Sudol and Hunter 2000); an Src homology domain 3 binding motif (SH3 BM) PVKQPPPLAP; a coiled-coil domain (CC); a C-terminal capped by TWL sequence, a PDZ domain ligand.

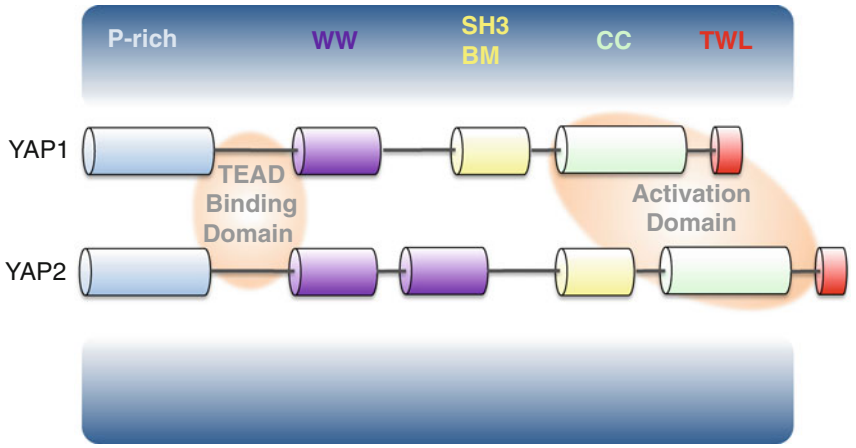

Fig. 8.3 Schematic representation of YAP protein. YAP gene encodes for two isoforms: YAP1 and YAP2. The main differences between YAP1 and YAP2 consist in the presence of 1 or 2 WW domains, respectively. YAP is a 65 kDa protein with several distinct domains: a proline-rich region at the N-terminal, one or two tandem WW domain in the middle followed by an Src homology domain 3 binding motif (SH3 BM), a coiled-coil domain (CC), and a C-terminal capped by TWL sequence, a PDZ domain ligand. The N-terminal is responsible for the interaction with the TEAD transcription factors family, and the C-terminal rich in serine, threonine, and acidic residues was shown to be a strong transcription activator

The N-terminal of YAP was mapped to be the TEAD family transcription factors interaction domain, and the C-terminal of YAP rich in serine, threonine, and acidic residues was shown to be a strong transcription activator.

8.2.5 Role in Animal Development

Different studies highlight the role of YAP in the control of organ growth. In fact, it was demonstrated that the disruption of the Hippo signaling pathway in *Drosophila* M. has a big impact in the growth of imaginal discs (Pan 2007). Moreover, YAP protein mutations are correlated to abnormal size and shape of fly wings (Zhao et al. 2007). Also in mice YAP has been demonstrated to play a pivotal role in cell growth control. YAP overexpression in mice liver induces a reversible increase of its size (Camargo et al. 2007; Dong et al. 2007).

At the molecular levels, the effects of YAP overexpression seem to depend on the inhibition of cell differentiation, partially by stimulating the Notch signaling pathway causing an expansion of multipotent-undifferentiated cells that maintain their ability to differentiate upon YAP gene expression inactivation (Camargo et al. 2007).

The involvement of YAP in the control of bone homeostasis and inhibition of osteoblast activity has also been demonstrated (Zaidi et al. 2004).

8.2.6 YAP in Tumorigenesis

From *Drosophila* M. genetic studies, the first clues about important roles of YAP in tumorigenesis was shown. In these studies ectopic expression of Yorkie leads to an increase in cell proliferation and tissue overgrowth (Huang et al. 2005). Consistent with this, YAP expression is elevated in gastric adenocarcinoma (Lam-Himlin et al. 2006) and the gene is amplified in liver cancer (Zender et al. 2006) and in mouse mammary tumors (Overholtzer et al. 2006). Furthermore, YAP and TAZ are highly expressed in a wide spectrum of human cancer cell lines and various primary tumors (Chan et al. 2008; Dong et al. 2007) supporting these two proteins as oncogenes. In non-transformed mammary epithelial cells, overexpression of YAP induces epithelial-to-mesenchymal transition, suppression of apoptosis, growth factor-independent proliferation, and anchorage-independent growth (Overholtzer et al. 2006). Likewise, the overexpression of TAZ triggers MCF10 mammary epithelial cells to undergo morphological changes, resembling cell transformation (Chan et al. 2008; Lei et al. 2008). Ectopic expression of TAZ also induces cell proliferation, overcomes contact inhibition, and leads to tumorigenesis in nude mice (Lei et al. 2008). Similarly, overexpression of YAP causes loss of contact inhibition (Dong et al. 2007). Other additional studies support the oncogenic role of YAP (Hao et al. 2008; Overholtzer et al. 2006), whereas other works support the idea that YAP might be a tumor suppressor by interacting with p73 (Matallanas et al. 2007; Oka et al. 2008; Strano et al. 2001), and is important for c-Jun-dependent apoptosis (Danovi et al. 2008). Moreover, a recent study showed that YAP acts as tumor suppressor in breast cancer (Yuan et al. 2008), despite being oncogenic when it is overexpressed in the mammary cell line MCF10A (Overholtzer et al. 2006).

8.2.7 YAP and its Role in Transcription

At the functional level, YAP serves as a coregulator for various transcription factors. YAP shares some transcription factor partners, including TEFs/TEADs (Vassilev et al. 2001) and RUNX proteins (Yagi et al. 1999; Zaidi et al. 2004). In addition, YAP is also known to interact with p73 (Basu et al. 2003; Levy et al. 2008; Strano et al. 2001, 2005). Among all these targets, only the interaction with the TEFs/TEADs appears to be conserved from fly to humans (Vassilev et al. 2001).

The domain of YAP responsible for the interaction with TEFs/TEADs transcription factors is localized at the N-terminal of YAP; it binds to the C-terminal domain of TEAD2/TEF4 (Vassilev et al. 2001; Zhao et al. 2007).

Interestingly, TEAD1 and TEAD2 double knockout mice display similar phenotypes as Yap knockouts (Sawada et al. 2008).

Another important interactor of YAP is the transcription factor RUNX2, which exerts a significant role in skeletal mineralization because it stimulates osteoblast differentiation of mesenchymal stem cells, promotes chondrocyte hypertrophy, and contributes to endothelial cell migration and vascular invasion of developing bone. Like other RUNT domain proteins, RUNX2 is a context-dependent transcriptional activator and repressor of genes that regulate cellular proliferation and differentiation.

YAP was identified as a binding partner of mammalian RUNX proteins in yeast two-hybrid screens wherein the proline-rich activation domain of RUNX1 was used as bait (Yagi et al. 1999). YAP was subsequently shown to interact with full-length RUNX2 in osseous cells via co-immunoprecipitation of endogenous proteins and co-immunofluorescence (Zaidi et al. 2004). RUNX2 recruits YAP to subnuclear foci and to the osteocalcin gene promoter, but does not affect its nucleo-cytoplasmic shuttling (Zaidi et al. 2004). The Y residue in the PPPYP motif of RUNX2 is essential for the interactions with YAP (Zaidi et al. 2004). There is no indication showing that YAP can bind to DNA, but when fused to the GAL4-DBD, YAP acts as a transcriptional coactivator of a heterologous GAL-dependent reporter (Yagi et al. 1999; Zaidi et al. 2004). Accordingly, YAP doubled the RUNX2 (PEBP2aA1)-dependent activation of the IgC-α promoter in p19 cells and a dominant-negative of YAP construct blocked RUNX2-dependent activation of osteocalcin promoter in NIH3T3 cells. However, a full-length version of YAP was not tested on the osteocalcin promoter (Yagi et al. 1999). Zaidi et al. (2004) showed that YAP repressed RUNX2-dependent activation of the osteocalcin promoter in NIH3T3 cells and four other cell lines. Thus, YAP-mediated repression of RUNX2 activity on the osteocalcin promoter is cell-type independent. YAP-mediated repression of RUNX2 instead seems to be dependent on promoter context: YAP blocks RUNX2-dependent activation of TGFβR1 promoter and enhances RUNX2-dependent repression of its own promoter. However, it does not affect RUNX2 transcriptional effects on the p6OSE2 or p21 promoters (Zaidi et al. 2004). These data indicate that RUNX2 can recruit YAP to promoter regions, but the effects of YAP on the expression of RUNX2 target genes is dependent on the cohort of other DNA binding proteins and co-factors brought to the gene by specific DNA sequences and protein–protein interactions. Transcriptional repression of RUNX2 by YAP is dependent on Src-induced activation and phosphorylation of YAP (Zaidi et al. 2004). Tyrosine phosphorylation of YAP is required for its subnuclear co-localization with RUNX2 but not for its nucleo-cytoplasmic transport (Zaidi et al. 2004). Thus, YAP is a signal-responsive and context-dependent regulator of RUNX2 activity that may facilitate gene expression in response to extracellular signals or oncogene activation.

8.3 YAP and p73

8.3.1 YAP Is a Transcriptional Coactivator of p73

Looking for p73 interacting proteins, it was found by Strano et al., that YAP1 was able to bind through its WW domain a PY motif present on the C-terminal region of

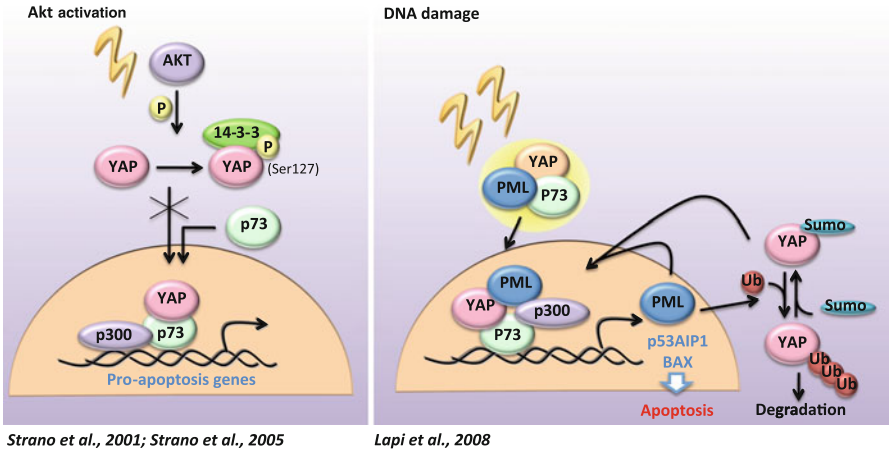

Fig. 8.4 YAP and p73 functions in apoptosis. YAP and p73 cooperate with each other to promote apoptosis upon DNA damage. In particular, YAP exerts an important role in the regulation of p73 stability and transcriptional activity by promoting p300 recruitment on p73 target genes. As shown in the *left panel* Akt-mediated phosphorylation of YAP protein has a negative effect on p73 transcriptional activity because it causes 14-3-3 binding to YAP and its consequent retention into the cytoplasm. In the *right panel*, the pro-apoptotic autoregulatory feedback loop is represented in which PML, YAP, and p73 are involved. Upon DNA damage, PML promotes YAP recruitment into the nuclear bodies where it binds to and activates p73. The YAP/p73 complex protein binds to the promoters of the apoptotic target genes recruiting also p300 and thus promoting their transcriptional activation. Among the target genes there is also PML, that in turn promotes the pro-apoptotic activity of YAP/p73 complex by favoring its stability

p73 (Strano et al. 2001). All the members of the family were checked for this interaction and the shorter members, p73γ and p63γ, and p53 themselves were excluded from the list. Therefore, YAP seems to be selective in choosing its partners among this family of transcription factors, a choice that may guide cells toward a defined outcome when they need YAP function. In 2005, Strano et al., demonstrated that p73 is required for the nuclear translocation of endogenous YAP in cells exposed to cisplatin and that YAP is recruited by PML gene into the nuclear bodies to promote p73 transcriptional activity (Strano et al. 2005). They found that YAP contributes to p73 stabilization in response to DNA damage and promotes p73-dependent apoptosis through the specific and selective coactivation of apoptotic p73 target genes and reinforcement of p300-mediated acetylation of p73 (Fig. 8.4, left panel). Altogether, these results identify YAP as an important determinant for p73 target gene specificity through p300 recruitment and p73 acetylation. In fact, using chromatin-immunoprecipitation, it was shown that upon DNA damage the complex PML-YAP-p73 recruits the acetyl-transferase p300 and together go into the regulatory regions of pro-apoptotic genes and upregulate their transcription (Strano et al. 2005).

Moreover, a YAP effect on p73 protein stability has been demonstrated due to the ability of YAP to bind the same region of p73 usually bound by the ubiquitin-protein ligase Itch. In this way, YAP prevents proteasome-mediated degradation of p73.

8.3.2 *YAP, p73, and PML Are Involved in a Pro-apoptotic Autoregulatory Feedback Loop*

Another important player in the YAP-p73 axis is the PML tumor suppressor gene.

The PML gene, involved in the chromosomal translocation t(15;17) of acute promyelocytic leukemia (APL), encodes a protein that localizes to the PML-nuclear bodies, that has been shown to play an important role in growth suppression, apoptosis, and senescence. In addition, it is induced upon different cellular stress signals and pro-apoptotic stimuli, such as ionizing radiations (Ferbeyre et al. 2000; Pearson et al. 2000). Its tumor suppressor activity has also been demonstrated in PML$^{-/-}$ mice: they present resistance to the lethal effects of γ radiations and to apoptosis induced by CD95 (Salomoni and Pandolfi 2002).

As mentioned before, Strano et al., in 2005 showed how PML is also important in p73-mediated apoptosis in response to cisplatin treatment (Strano et al. 2005).

Afterwards in 2008, Lapi et al. emphasized the existence of an autoregulatory feedback loop in which PML, YAP, and p73 are involved (Fig. 8.4, right panel) (Lapi et al. 2008). In more detail, by using a gene expression microarray analysis in human colon cancer cells HCT116 treated with cisplatin, they found that PML expression is positively modulated by the protein complex p73/YAP. PML is not a specific p73 target gene, since it is controlled by p53 (de Stanchina et al. 2004). In fact, the authors demonstrated how p53 can synergize with p73/YAP complex in the transcriptional regulation of PML during the apoptotic response of HCT116 cells. However, they showed that in particular cellular contexts, in which p53 is not present or mutated, p73 is still able to induce PML expression and nuclear bodies formation. Moreover, by using a constitutively active mutant of AKT that restrains YAP into the cytoplasm, they stressed the important role of YAP as a coactivator of p73 in the transactivation of PML.

The existence of an autoregulatory feedback loop is due to the ability of PML to control YAP stability. In fact, the authors proved the presence of a physical interaction taking place between the WW domain of YAP and the PVPVY motif of PML. This interaction has an effect on YAP half-life since it promotes YAP sumoylation that prevents YAP ubiquitination and therefore its proteasomal degradation.

PML upregulation by p73/YAP complex has an effect also on p73 transcriptional activity itself, in fact, as mentioned before, PML promotes p300-mediated acetylation of p73, that in turn induces the p73 pro-apoptotic response (Bernassola et al. 2004).

8.4 Conclusions

It is becoming increasingly clear that the efficiency and the efficacy of a tumor suppressor response can also be based on the number of engaged tumor suppressor pathways. The existence of an autoregulatory feedback loop between YAP, p73, and

PML, which closely links three different tumor suppressor pathways, might hold promise for anticancer therapeutic approaches. YAP which appears to bridge p53 and PML tumor suppressor activities might turn to be an intriguing and potentially attractive therapeutic target whose modulation maximizes distinct tumor suppressor activities.

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Chapter 9
The c-Abl/YAP/p73 Apoptotic Module and the HIPPO Pathway

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Abstract The HIPPO pathway is an evolutionarily conserved pathway that regulates cell proliferation and organ size. The canonical pathway is triggered by cell–cell contact, which leads to a series of signaling events that culminate in the nuclear exclusion of the downstream effectors, the pro-proliferative transcription coactivators YAP and TAZ. However, while the canonical role of YAP and TAZ is to promote proliferation, DNA damage leads to a switch in the role of YAP from pro-proliferative to pro-apoptotic. The mechanisms leading to YAP-mediated apoptosis will be discussed in this chapter, focusing on the role of the non-receptor tyrosine kinase c-Abl. c-Abl activity is needed for the switch of YAP from anti- to pro-apoptotic activity, as well as for the regulation of YAP and p73 accumulation. This switching mechanism introduces a certain level of complexity in our attempt to categorize onco- and tumor suppressor genes. p73, YAP, and TAZ are highly disordered proteins, an attribute of key regulatory proteins that interact with many partners. Disordered proteins undergo proteasomal degradation through both ubiquitin-dependent and -independent mechanisms. This double mechanism ensures an optimal HIPPO pathway proteostasis.

Keywords YAP • p73 • c-Abl • Apoptosis • Default degradation • 20S proteasome • Ubiquitin-independent degradation • Nanny model • IDP • Intrinsically disordered protein

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_9,

9.1 Introduction

The canonical HIPPO pathway controls cell proliferation and organ size through inhibition, via phosphorylation by LATS, of the transcription coactivators YAP and TAZ. However, noncanonical roles have also been established for these players, and thus these activities must be considered when approaching the system as a whole. The YAP coactivator can play diametrically opposed roles depending on its transcription factor partner. YAP association with the TEAD family of transcription factors leads to transcription of genes promoting cell proliferation, epithelial-to-mesenchymal transition (EMT), inhibition of apoptosis, and tumorigenesis. In contrast, YAP association with p73, a p53 tumor suppressor paralog, promotes apoptosis. In this chapter, we will review the apoptotic pathway mediated by YAP and p73, and discuss how regulation by the non-receptor tyrosine kinase c-Abl leads to the switching of YAP from anti- to pro-apoptotic activity.

YAP and TAZ, like many other key regulators, are intrinsically disordered proteins. This attribute is needed for multiple protein–protein interactions and intensive protein modifications such as phosphorylation. Indeed the number of proteins identified as interacting with YAP and TAZ and the sites of YAP and TAZ modification has increased since their discovery. In addition to modification by phosphorylation, the downstream effectors of the HIPPO pathway are regulated at the level of protein accumulation. c-Abl regulates the stability of p73 and YAP. This is achieved through protection from ubiquitin-dependent proteasomal degradation. However, YAP and p73 are also substrates of ubiquitin-independent degradation, and c-Abl stabilization of YAP and p73 may also be mediated through this pathway. Although regulation of protein levels by ubiquitin-independent degradation is often overlooked, recently the importance of this mode has garnered increased recognition. We will discuss how the ubiquitin-independent degradation pathway should be considered when studying the HIPPO pathway, which includes several known and predicted substrates.

9.2 YAP and p73 in Apoptosis

YAP interacts with many partners via distinct domains. Several of the partners are transcription factors, including the TEAD/TEF (Vassilev et al. 2001) and RUNX (Yagi et al. 1999; Zaidi et al. 2004) families. Association of YAP with TEAD/TEF leads to the transcription of genes that promote proliferation, EMT, tumorigenesis, stem cell renewal, and inhibition of apoptosis (Lian et al. 2010; Zhao et al. 2008a; Zhang et al. 2009; Ota and Sasaki 2008; Sawada et al. 2008). YAP association with RUNX leads to transcription from promoters such as osteocalcin (Yagi et al. 1999; Zaidi et al. 2004) and Itch (Levy et al. 2008a). In 2000, the Yaffe group noted that a number of transcription factors including p73 bear the sequence motif, PPxY, capable of binding the WW domain of YAP and TAZ (Kanai et al. 2000). In 2001, Strano

et al. (2001) reported that YAP associates with p63 and p73, the p53 paralogs. Since the interaction is mediated through the YAP WW domain and the PPPPY motif of p73, YAP interacts only with the long isoforms p73α, p73β, and p63α, which contain the PPPPY motif, and not with p73γ. RUNX also interacts with YAP through the YAP WW domain (Yagi et al. 1999), but the interaction of YAP with TEAD does not rely on the WW domain. Rather, this interaction is based on a TEAD-binding domain at the N-terminus of YAP, and a C-terminal region in TEAD (Vassilev et al. 2001; Li et al. 2010).

In contrast to the target genes induced by YAP/RUNX or YAP/TEAD, association of YAP with p73 leads to a dramatically different outcome. YAP functions as a coactivator of p73 (Strano et al. 2001), and YAP is important for the induction of apoptosis in response to DNA damaging agents (Basu et al. 2003; Levy et al. 2008b; Strano et al. 2005; Hamilton et al. 2009). YAP association with p73 imparts selectivity in p73 apoptotic targets, leading to transcription of pro-apoptotic targets such as BAX and p53AIP1, rather than the cell cycle arrest target p21 (Strano et al. 2005). In contrast to TEAD that bears a weak transcription activation domain (TAD), p73 contains a strong TAD; therefore, the role of YAP as a transcription coactivator of p73 might not be the whole story. Indeed YAP in the context of p73 plays some other roles as well, such as inhibiting p73 degradation (see below).

P73 and YAP are regulated at the level of subnuclear compartmentation. PML (promyelocytic leukemia protein) is a major organizer of PML nuclear bodies, which serve a scaffolding and regulatory role for cellular processes including apoptosis, senescence, DNA repair, and antiviral defense (Lallemand-Breitenbach and de The 2010; Bernardi et al. 2008; Salomoni et al. 2012). PML is needed for the apoptotic response by p73/YAP (Strano et al. 2005). PML interacts with p73, and inhibits ubiquitin-dependent degradation of p73. This is mediated through p73 acetylation by p300, which is dependent on PML (Bernassola et al. 2004). Interestingly, PML bodies are also needed for YAP coactivation of p73. Coactivation of p73 by YAP is dependent upon PML and localization to nuclear bodies, and YAP also contributes to the accumulation of p73 in response to DNA damage, and its acetylation by p300 (Strano et al. 2005). However, the underlying mechanisms of how p73 and YAP target PML have not yet been resolved, neither has the PML function in this process. PML is conjugated by a ubiquitin-like protein named SUMO. PML conjugation by SUMO plays a critical role in recruitment of proteins, many of which are sumoylated as well (reviewed in Lallemand-Breitenbach and de The 2010). The question of whether YAP and p73 undergo this modification prior to PML association, although important to substantiate the role of PML in this process, remains open.

YAP regulation of p73-dependent apoptosis and p73 levels does not occur spontaneously; rather, it occurs in response to DNA damage. Thus, DNA damage causes a switch in YAP activity, from promoting proliferation to promoting apoptosis. This switch is mediated through the non-receptor tyrosine kinase c-Abl, which is activated by DNA damage.

9.3 The Non-receptor Tyrosine Kinase c-Abl; Domain Structure and Modes of Activation

c-Abl is ubiquitously expressed in mammalian cells, and has both cytoplasmic and nuclear activities (Shaul and Ben-Yehoyada 2005; Pendergast 2002; Colicelli 2010). c-Abl possesses both NLS and NES motifs, and is thought to shuttle between nucleus and cytoplasm based on environmental signals, such as cell adherence to solid substrates (Taagepera et al. 1998). Human c-Abl has two alternatively spliced forms, 1a and 1b. The 1b isoform has a myristoylation site, which allows for membrane association, and is also involved in regulation of c-Abl autoinhibition (Nagar et al. 2003; Hantschel et al. 2003). The N-terminal region of c-Abl has several defined domains of the Src kinase family (reviewed in Pendergast 2002; Colicelli 2010) (Fig. 9.1). The SH3 (Src homology domain 3) binds to proline-rich sequences, with the consensus being PXXP. This domain is followed by an SH2 domain that preferentially binds phosphotyrosine residues. The tyrosine kinase domain (SH1) is followed by a unique long C-terminus. The C-terminal region contains proline-rich domains, the NES and NLS motifs, DNA-binding domain, and domains for binding to F- and G-actin. C-Abl folds into an autoinhibitory conformation, where the kinase domain is shielded by the SH3 and SH2 domains, and the conformation is secured by the interaction of the myristoylated N-terminus with the kinase domain (Pluk et al. 2002; Nagar et al. 2003).

Activation of c-Abl is achieved by opening of the autoinhibitory conformation. In the case of the constitutively active oncogenic forms of ABL, such as BCR-ABL, translocation creates a fusion protein where the N-terminus of BCR is fused to ABL, which prevents the folding into the autoinhibitory conformation. Activation of wild-type c-Abl is achieved through phosphorylation of the activation loop, either by other kinases, such as Src, or by autophosphorylation (reviewed in Colicelli 2010; Pendergast 2002). Phosphorylation of Y412 in the activation loop, and at Y245 in the kinase domain, is needed for full activation of c-Abl (Brasher and Van Etten 2000). C-Abl can also be activated by binding to adaptor proteins, such as the SH3-domain containing proteins Nck (Smith et al. 1999) and Crk (Shishido et al. 2001), which interact with proline-rich regions in the C-terminus of c-Abl. These and other proteins, which are also substrates of c-Abl, are involved in c-Abl regulation of actin dynamics, which is important for neural growth cone formation, cytoskeletal organization, and cell motility (reviewed in Pendergast 2002; Colicelli 2010).

C-Abl regulates cell proliferation and is regulated by mitogenic signals. C-Abl localized to the cell membrane is activated by growth factors EGF and PDGF, and this is mediated through Src kinases and phospholipase C-γ (Plattner et al. 1999, 2003). Both the effect of PDGF on actin dynamics, seen as increased dorsal membrane ruffling, and PDGF mitogenic capacity, are mediated through c-Abl (Plattner et al. 1999). Interestingly, while c-Abl is activated by PDGFR and promotes PDGF-mediated migration and proliferation, c-Abl phosphorylation of PDGFR acts in a negative feedback mechanism to inhibit PDGFR-mediated chemotaxis (Srinivasan

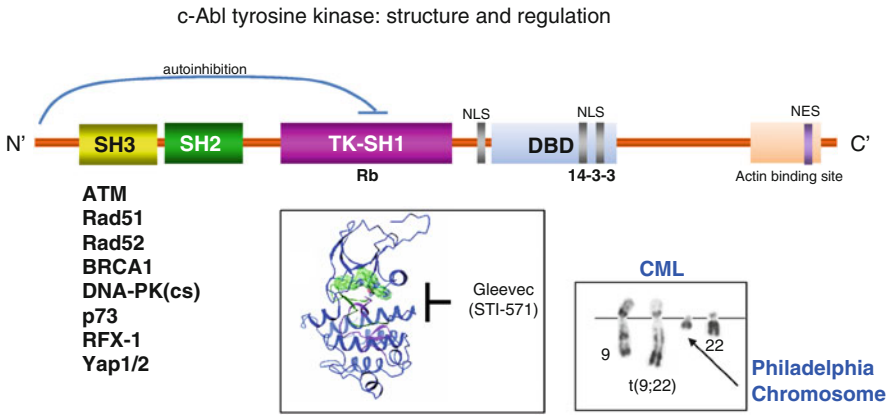

Fig. 9.1 c-Abl tyrosine kinase, structure and regulation. Schematic representation of c-Abl tyrosine kinase. *SH3* Src homology domain 3; *SH2* Src homology domain 2; *TK-SH1* tyrosine kinase, Src homology domain 1; *NLS* nuclear localization signal; *DBD* DNA-binding domain; *NES* nuclear export signal; and binding sites for actin are shown. Listed below are some of the proteins involved in DNA damage response shown to bind to the conserved domains. The structure showing interaction of the c-Abl inhibitor, Gleevec, with the c-Abl kinase domain is shown below the kinase domain. Also shown is the translocation involving ABL on chromosome 9 and BCR on chromosome 22, resulting in the Philadelphia chromosome, which causes chronic myelogenous leukemia, CML

et al. 2009). C-Abl is inhibited by binding of Rb to the kinase domain. As the cell moves from G1 to S phase, c-Abl is freed from Rb, and becomes activated (Welch and Wang 1993). While nuclear c-Abl is activated in S phase, the activity of cytoplasmic c-Abl is not affected by the cell cycle.

c-Abl is also active in stress response. Cytoplasmic c-Abl is activated by oxidative stress in a mechanism involving PKC-δ (Sun et al. 2000a, b) and in c-Abl deficient cells, H_2O_2-induced apoptosis is attenuated (Sun et al. 2000a). Compounds that induce ER stress, including tunicamycin, brefeldin A, or the calcium ionophore A 2318 activate c-Abl, and lead to translocation of ER-associated c-Abl to the mitochondria. Furthermore, apoptosis induced by ER stress is reduced in c-Abl-deficient cells (Ito et al. 2001). Oxidative and ER stress lead to apoptosis through release of mitochondrial cytochrome C in a c-Abl-dependent mechanism (Sun et al. 2000a; Ito et al. 2001).

C-Abl can translocate between the nucleus and the cytoplasm. However, phosphorylation of c-Abl by TTK/Mps1 on T735 (Nihira et al. 2008), creates a binding site for 14-3-3 (Yoshida et al. 2005), and leads to cytoplasmic localization of c-Abl. The T735 phosphorylation site is located between the second and third NLSs of c-Abl, and binding to 14-3-3 may mask the NLSs, impeding the nuclear localization of c-Abl (Yoshida 2007). Upon DNA damage, activation of JNK leads to the phosphorylation of 14-3-3, and the release of c-Abl, allowing the nuclear accumulation of c-Abl (Yoshida et al. 2005). Although YAP and TAZ are also sequestered in the cytoplasm through binding to 14-3-3 proteins (Kanai et al. 2000; Basu et al. 2003;

Zhao et al. 2007), it is not known whether JNK phosphorylation of 14-3-3 can lead to the nuclear localization of YAP and TAZ.

DNA damage by ionizing radiation and genotoxic agents leads to activation of nuclear c-Abl (Kharbanda et al. 1995a; Liu et al. 1996). This involves phosphorylation of c-Abl S465 by ATM (Baskaran et al. 1997; Shafman et al. 1997). Activation of c-Abl also relies on an intact mismatch-repair system, as c-Abl activation caused by different DNA damaging agents is impaired in cells deficient for mismatch repair (Nehme et al. 1999; Gong et al. 1999). The nonhomologous end joining (NHEJ) DNA repair protein DNA-PK also contributes to c-Abl activation (Kharbanda et al. 1997; Tang et al. 2012). Interestingly, c-Abl also phosphorylates, and is required for the full activation of ATM and ATR (Wang et al. 2011). Furthermore, c-Abl plays a role in activating JNK/SAPK and p38 MAP kinase pathways in response to DNA damage (Kharbanda et al. 1995a, b, 2000; Pandey et al. 1996). C-Abl has been shown to phosphorylate DNA-PK (Kharbanda et al. 1997) and other DNA damage response proteins, including RAD51 (Yuan et al. 1998), RAD52 (Kitao and Yuan 2002), and WRN (Cheng et al. 2003). In the case of DNA-PK, RAD51, and WRN, c-Abl phosphorylation inhibits their activities. This inhibitory activity toward DNA damage repair proteins is consistent with the finding that c-Abl inhibits the slow phase of DNA repair (Meltser et al. 2010). Meltser et al. showed that following ionizing radiation, most repair of double-strand breaks is concluded within 1–2 h, whereas breaks remaining after that period are repaired much more slowly (Meltser et al. 2010). C-Abl plays a role in down-regulating this later phase of repair, which is assumed to be less accurate than in the initial stage. One interpretation of this activity is that this paves the way for induction of apoptosis in cells with low likelihood of complete and accurate repair of their DNA. In this situation, active c-Abl is then poised to induce apoptosis, through activation of the p53-family member p73, and its coactivator YAP.

9.4 Regulation of p53 Family Proteins by c-Abl Tyrosine Kinase

The non-receptor tyrosine kinase c-Abl plays multiple roles in the regulation of the apoptotic pathway. Although later work showed that this role is primarily mediated through p73, early studies made connections between c-Abl and p53. Sawyers et al. demonstrated that nuclear expression of c-Abl led to cell cycle arrest, which was reminiscent of control by Rb and p53 (Sawyers et al. 1994). This group then showed that c-Abl bound p53, and c-Abl-induced cell cycle arrest was p53-dependent (Goga et al. 1995). Furthermore, c-Abl-mediated DNA damage-induced cell cycle arrest was shown to be dependent on p53 (Yuan et al. 1996a, b). C-Abl can phosphorylate Hdm2, the p53 E3 ligase, and the related p53 inhibitor Hdmx, and this inhibits their activities. In this way, c-Abl supports p53 accumulation and function (Zuckerman et al. 2009; Sionov et al. 1999; Goldberg et al. 2002).

However, c-Abl-dependent apoptotic response to DNA damage was found to occur in cells deficient for p53, indicating that c-Abl could operate through another mechanism (Yuan et al. 1997). This mechanism is mediated through the p53 family members p73, and p63 under certain cell contexts.

C-Abl activation affects the p73-dependent apoptotic response via several mechanisms. In response to DNA damage, activated c-Abl directly phosphorylates p73, which leads to p73 stabilization, and is needed for p73 apoptotic activity (Agami et al. 1999; Gong et al. 1999; Yuan et al. 1999). In contrast, c-Abl does not directly phosphorylate p53 (Ben-Yehoyada et al. 2003). P73 is phosphorylated by c-Abl on Y99 (Yuan et al. 1999), and the interaction between the c-Abl SH2 domain and phosphorylated p73 is needed for p73 stabilization (Tsai and Yuan 2003). Interaction between c-Abl and p73 depends on the c-Abl SH3 domain and a PxxP motif located in the linker region of p73 (Agami et al. 1999). Phosphorylation of p73 by c-Abl leads to the association of p73 with the nuclear matrix (Ben-Yehoyada et al. 2003). As PML is associated with the nuclear matrix (Lallemand-Breitenbach and de The 2010), c-Abl phosphorylation of p73 leads to increased association with PML, which, as mentioned above, leads to increased p73 stabilization due to PML-dependent acetylation (Bernassola et al. 2004), and increased apoptotic activity (Strano et al. 2005). Additionally, the prolyl isomerase Pin1, which recognizes phosphorylated serine or threonine residues that are followed by proline, is needed for stabilization of p73, and this mechanism is dependent upon c-Abl (Mantovani et al. 2004). Following DNA damage, c-Abl phosphorylation of p73 leads to subsequent phosphorylation of p73 by p38 MAPK (Sanchez-Prieto et al. 2002). P73 phosphorylated by p38 binds to Pin1, and this enhances acetylation of p73 by p300, and p73 stabilization (Mantovani et al. 2004).

9.5 Regulation of YAP/p73-Mediated Apoptosis by c-Abl Tyrosine Kinase

In response to DNA damage, c-Abl also phosphorylates YAP, at Y357 (Y391 in YAP2), leading to an increase in YAP levels (Levy et al. 2008b). The mechanism by which c-Abl phosphorylation increases YAP levels has not been revealed yet, but it may be achieved via two distinct mechanisms. The first involves YAP degradation via β-TrCP. YAP phosphorylation by LATS on S381 leads to subsequent phosphorylation by CK1δ/ε, which marks the YAP phosphodegron as a substrate for β-TrCP-mediated ubiquitination, which is then followed by proteasomal degradation (Zhao et al. 2010). The c-Abl phosphorylation site, Y391, is adjacent to the DSG phosphodegron, leading to the possibility that phosphorylation of YAP by c-Abl inhibits YAP degradation by interfering with the association/phosphorylation of YAP by LATS, CK1δ/ε, or with β-TrCP (Fig. 9.2). TAZ degradation is also mediated through a β-TrCP. However, in contrast to YAP, there are two consensus DSG phosphodegron motifs, with the C-terminal motif being more similar to the YAP DSG. Both

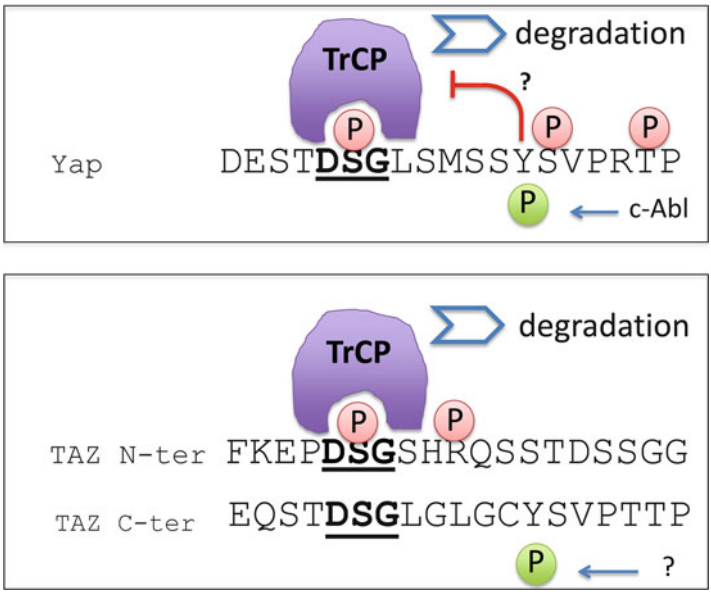

Fig. 9.2 YAP and TAZ phosphodegron motif. The phosphodegron DSG motif recognized by β-TrCP and surrounding sequence is shown. The tyrosine residue in YAP phosphorylated by c-Abl is indicated. TAZ has two consensus DSG motifs. The N-terminal motif has been shown to be the one targeted by β-TrCP in degradation of TAZ. Interestingly, the C-terminal motif is more similar to the YAP DSG motif, and possesses a putative tyrosine phosphorylation site

play a role in regulating TAZ levels under different conditions (Liu et al. 2010; Huang et al. 2012). In addition to potential modulation of β-TrCP-mediated YAP degradation, c-Abl phosphorylation of YAP may help in preventing YAP degradation through the ubiquitin-independent default degradation pathway (see below).

C-Abl phosphorylation not only increases YAP levels, but it also leads to an increased association of YAP with p73, leading YAP to preferentially associate with p73 instead of RUNX. The increased level of YAP, and its increased affinity for p73, enable the stabilization of p73 by YAP by preventing ubiquitination by the E3 protein ligase Itch, as described below (Levy et al. 2007; Danovi et al. 2008). Furthermore, YAP phosphorylated by c-Abl accumulates in the nucleus, and specifically associates with p73 on pro-apoptotic targets, such as Bax and PIG3, at the expense of non-apoptotic p73 targets, such as p21 (Levy et al. 2008b). Likewise, DNA damage-induced phosphorylation of YAP by c-Abl causes YAP to dissociate from RUNX, a regulator of Itch, the p73 E3 ligase. In this way, c-Abl enables the switching of YAP toward an apoptotic program (Fig. 9.3). It is, however, not clear whether under this condition tyrosine phosphorylated YAP is in association with TEAD transcription factors. Phosphomimetic YAP mutant is as active as wild-type

YAP in coactivating TEAD major target genes, suggesting that YAP modification is unlikely to modify the TEAD transcription program. Remarkably however, this YAP mutant is inactive in inducing cell transformation (unpublished observation), suggesting that the function of the new targets of the tyrosine phosphorylated YAP, such as those activated by p73 is dominant over the function of the YAP-TEAD transcription program.

The molecular mechanism of the c-Abl-mediated YAP target switch is still unknown. Particularly challenging is understanding the mechanism of YAP dissociation from RUNX at the level of the target genes. This is because it is unlikely that the whole YAP pool undergoes c-Abl-mediated tyrosine phosphorylation and the residual unmodified YAP should remain RUNX associated. The simplest explanation is that c-Abl directly targets the promoter-associated RUNX-YAP complex, although this remains to be shown experimentally. The fact that c-Abl is in association with promoters/enhancers has been demonstrated by a few studies. c-Abl binds to specific DNA sequences (Dikstein et al. 1992), and c-Abl that is associated with DNA is preferentially phosphorylated and activated (Dikstein et al. 1996). Also, a role for c-Abl has been shown in enhancing transcription through the phosphorylation of the CTD of RNA polymerase II (Baskaran et al. 1993, 1996). However, a role for this phosphorylation in induction of specific target genes is not known.

9.6 Regulation of HIPPO Pathway Proteostasis

A critical mode of regulation relies on mechanisms of protein homeostasis (proteostasis). The HIPPO pathway effectors are labile proteins, an attribute shared by many important regulators. Therefore, for better understanding of this pathway one needs to know the mechanisms of their degradation and how this process is modulated under different physiological conditions. Our knowledge on p73 is quite good. P73 is degraded in a ubiquitin-dependent manner, mediated by the ubiquitin ligase Itch (Rossi et al. 2005). P63, the p73 paralog, is also a substrate of Itch, and interaction is mediated in a manner analogous to the Itch/p73 interaction (Rossi et al. 2006). The interaction between p73 and Itch is mediated through the p73 PPPPY domain and Itch WW domains (Rossi et al. 2005). Notably, YAP also interacts with p73 through binding of YAP's WW domain to the p73 PPPPY motif. Thus, YAP-induced accumulation of p73 is due to its ability to compete with Itch for the binding to p73, thus preventing ubiquitination of p73 by Itch (Levy et al. 2007; Danovi et al. 2008) (Fig. 9.3). A similar mechanism would be predicted for protection of p63. In addition, c-Abl phosphorylation of YAP is involved in down-regulating Itch expression under DNA damage, as described in the previous section.

Interestingly, the HIPPO kinase LATS1, but not LATS2, is also a substrate of Itch (Salah et al. 2011; Ho et al. 2011). It is not yet known whether YAP can also protect LATS1 from Itch-dependent degradation, as it does for p73. What has been shown is that Itch overexpression leads to a reduction in LATS1 levels, leading to

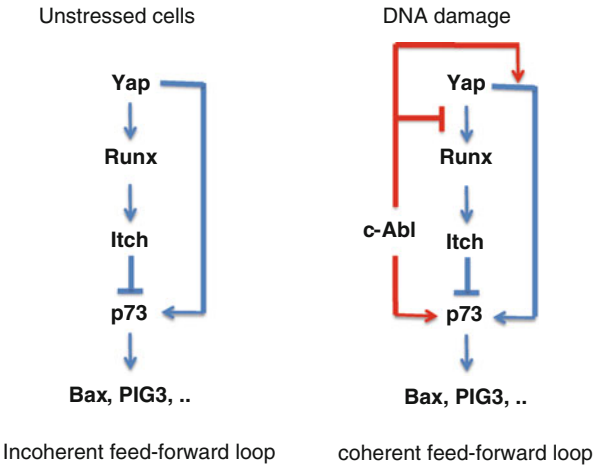

Fig. 9.3 Regulation of YAP/p73 mediated apoptosis. Under normal conditions, p73 undergoes proteasomal degradation mediated by the ubiquitin E3 ligase Itch. YAP coactivation of RUNX leads to higher levels of Itch, and reduced p73. Although YAP can also protect p73 from Itch, in the absence of DNA damage the net effect is p73 degradation. This represents an incoherent feed-forward loop. In the presence of DNA damage, c-Abl is activated, and this increases YAP levels and the propensity of YAP to associate with p73, rather than with RUNX. As a result, Itch levels are reduced, p73 levels increase, and p73/YAP activates pro-apoptotic target genes. Thus, activation of c-Abl switches the circuit to a coherent feed-forward loop

lower phosphorylation of YAP on S127, more nuclear localization of YAP, and more transcription of YAP pro-proliferative target genes. Knockdown of Itch leads to increased LATS1 and increased LATS-dependent apoptosis, as apoptosis in this situation was again decreased upon knockdown of LATS (Ho et al. 2011; Salah et al. 2011). Since Itch knockdown is also expected to lead to higher levels of p73, it is possible that apoptosis under this condition is also dependent on p73. This principle of regulation that is based on sequestration of the WW domain ligand sequence (PPxY, in the described examples) might have wider implications. For example, there are a relatively large number of WW domain containing proteins including a few other E3 ligases that their access to the substrate is eliminated by this mechanism (Shearwin-Whyatt et al. 2006).

Interestingly, YAP, by targeting RUNX, promotes the transcription of the Itch gene (Levy et al. 2008a). However, upon DNA damage YAP is phosphorylated by c-Abl, and this causes YAP to switch its association from RUNX in favor of p73. This leads to a reduction in transcription of Itch (Levy et al. 2008a). Thus, c-Abl also leads to the stabilization of p73 by reducing transcription of the E3 ligase Itch by disrupting the YAP-RUNX complex (Fig. 9.3). As LATS1 is also a target of Itch, it is predicted that activation of c-Abl by DNA damage would lead to an increase in LATS1 levels (Ho et al. 2011; Salah et al. 2011). However, this critical question has

not been challenged and an effect of DNA damage on levels of LATS has not been reported. The interactions of YAP, p73, Runx, Itch, and c-Abl can be summarized as shown in Fig. 9.3. In the absence of c-Abl phosphorylation, increased levels of YAP activate RUNX on the Itch promoter, leading to reduced p73 levels. This results in an incoherent feed-forward loop. In contrast, when YAP is phosphorylated by c-Abl, this causes YAP to dissociate from RUNX, leading to reduced Itch, and increased levels of p73. YAP also protects p73 from Itch-mediated degradation through binding to p73. Thus, in the presence of c-Abl phosphorylation of YAP, the circuit is transformed into a coherent feed-forward loop.

In the context of canonical activation of the HIPPO pathway by high cell density, MST and LATS ensure the nuclear exclusion of YAP. Yet, under other contexts, MST, LATS, Salvador, and RASSF1A promote apoptosis mediated by (nuclear) YAP and p73 (Matallanas et al. 2007; Donninger et al. 2011; Hamilton et al. 2009; Kawahara et al. 2008; Park et al. 2010; Yee et al. 2012). The details of this pro-apoptotic HIPPO cassette are presented in Chap. 7. Interestingly, MST1/2-promoted apoptosis is also subject to regulation by c-Abl. In response to oxidative stress in neurons, c-Abl phosphorylates MST1, leading to its stabilization and association with FOXO3, which then promotes apoptosis (Xiao et al. 2011). In addition, through a different mechanism, c-Abl is needed for MST2-mediated apoptosis. Here c-Abl phosphorylation of MST2 leads to its dissociation from Raf-1, enabling MST2 activation and induction of apoptosis (Liu et al. 2012).

YAP also associates with p63 (Strano et al. 2001). In oocytes, DNA damage leads to apoptosis that is dependent on c-Abl phosphorylation of p63 (Gonfloni et al. 2009). Full-length TA-p63 is implicated in this process, yet it is not known whether YAP plays a role in this response. Interestingly, c-Abl phosphorylation of the pro-survival ΔNp63 isoform leads to ΔNp63 stabilization (Yuan et al. 2010). Cisplatin treatment induces c-Abl phosphorylation of ΔNp63, and its association with YAP; however, these are implicated in protecting cells from cisplatin-induced cell death, rather than with inducing apoptosis, as is found with full-length p63 and p73.

In the previous sections we described mechanisms behind the DNA damage-induced stabilization of p73 and YAP. These mechanisms focused on escape from ubiquitin-dependent proteasomal degradation. The ubiquitin-proteasome system regulates the degradation of a vast array of cellular proteins, including those that are part of the HIPPO pathway. However, this system exists alongside ubiquitin-independent proteasomal degradation, a system that is often overlooked. While ubiquitin-dependent regulation requires modification of the substrate proteins by ubiquitin-E3 ligases, susceptibility of proteins to ubiquitin-independent degradation depends on the inherent characteristics of the given protein. Degradation of proteins by the proteasome necessitates a feeding of the protein into the catalytic core (20S particle) of the proteasome. The opening of this cylindrical core is small. Therefore, to enter the proteasome, proteins must present an unfolded region to the catalytic core of the proteasome (Smith et al. 2005; Kohler et al. 2001). Folded proteins are too large to enter the entry pore of the 20S proteasome. In the process of ubiquitin-dependent degradation,

folded, ubiquitinated proteins are recognized and bound by the 19S regulatory cap of the 26S proteasome. The cap structure has the ubiquitin-binding and deubiquitinase activities, as well as unfolding activity. Once it is deubiquitinated and unfolded, the protein can be fed into the 20S catalytic core of the proteasome, and degraded (Ruschak et al. 2010; Navon and Goldberg 2001). However, there are many examples of proteins that have regions that are intrinsically unstructured; their natural state is to be unfolded (Wright and Dyson 1999). These proteins are referred to as IDPs, intrinsically disordered proteins. Since these proteins possess unfolded regions, they are immediate substrates of the 20S proteasome, and require no modification prior to degradation. For this reason, degradation of these proteins occurs "by default" (Tsvetkov et al. 2009; Asher et al. 2006; Melo et al. 2011). Interestingly, many regulatory proteins are IDPs; their unstructured regions may give them more flexibility in terms of interaction with other proteins (Ward et al. 2004). In other words, the unstructured regions can be used to bind to different protein partners at different times, with the unstructured region adopting different conformations upon interacting with a specific protein partner. The interaction with other proteins also provides the means for regulating degradation by default. Interaction of an IDP with a protein partner protects the IDP from degradation by the 20S proteasome. The protein partner can be a permanent interacting protein, as in a functional complex, or to a more temporary binding protein, termed a "nanny" (Tsvetkov et al. 2009).

The ubiquitin-independent degradation pathway has shown to be a significant regulatory mechanism for many proteins, including those also degraded through ubiquitin-dependent means. These proteins include (but are not limited to) p53 (Asher and Shaul 2005, Asher et al. 2001, 2002a, b), ODC (Asher et al. 2005a), c-Fos (Adler et al. 2010), BIM(EL) (Wiggins et al. 2011), p21 (Touitou et al. 2001; Tsvetkov et al. 2008), BAF57 (Keppler and Archer 2010), thymidylate synthase (Melo et al. 2011), and others. Furthermore, p73 (Asher et al. 2005b), and the HIPPO proteins YAP and TAZ (Tsvetkov et al. 2012) are all subject to ubiquitin-independent degradation by default. YAP and TAZ are particularly sensitive to default degradation, when compared to other substrates of 20S proteasomal degradation (Tsvetkov et al. 2012). They possess a very high level of disorder. A schematic representation of YAP and TAZ disorder, as predicted by the program FoldIndex© (Prilusky et al. 2005) is shown in Fig. 9.4. As noted above, substrates of this type of degradation, degradation by default, can be protected from degradation by binding to a protein partner, or to a nanny (Asher et al. 2006). The protein NQO1 serves as a nanny for several IDPs, including p73 (Asher et al. 2005b), p53 (Asher et al. 2002b), and c-FOS (Adler et al. 2010). The interaction between YAP and p73, which is increased following c-Abl phosphorylation, may also serve to protect both proteins from default degradation. Thus, c-Abl phosphorylation may be affecting stability of YAP and p73 through this mechanism as well. There are other protein–protein interactions within the HIPPO network that may serve nanny functions for YAP, TAZ, or other HIPPO proteins. Interaction of YAP and TAZ with the 14-3-3 proteins (Basu et al. 2003; Zhao et al. 2007; Kanai et al. 2000), while serving

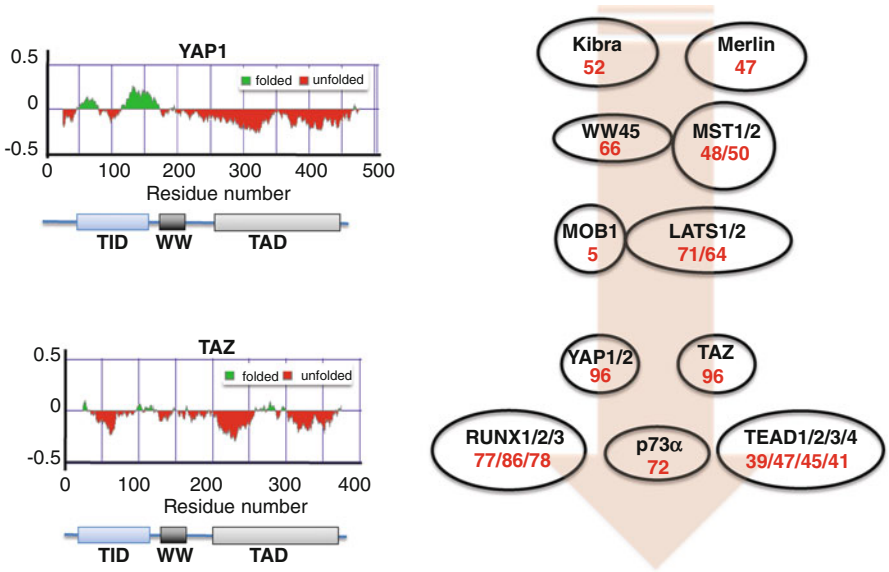

Fig. 9.4 Intrinsically disordered proteins of the HIPPO pathway. *Left panel*: Schematic representation of YAP and TAZ, and the predicted regions of disorder as analyzed by FoldIndex© (Prilusky et al. 2005). *Right panel*: Components of the HIPPO pathway are listed with the percentages of disordered regions, as calculated using IUPred (Dosztanyi et al. 2005a, b). The number represents the percent of amino acids in the protein with a disorder score above 0.4

to sequester them in the cytoplasm, may also be serving a nanny function, and allowing a protected reservoir of these proteins to remain undegraded. Similarly, YAP and TAZ interaction with LATS1/2 (Zhao et al. 2007; Huang et al. 2005; Hao et al. 2008; Lei et al. 2008), AMOT (Webb et al. 2011; Chan et al. 2011; Zhao et al. 2011) and ZO-2 (Oka et al. 2010; Remue et al. 2010), while having implications for cellular localization, may also be affecting YAP/TAZ levels by preventing default degradation. An important prediction is that the levels of YAP and TAZ change in direct correlation with the levels of the interacting proteins; namely the other HIPPO pathway components. Other HIPPO proteins may also be regulated through default degradation. Many of the HIPPO pathway proteins have disordered regions. Figure 9.4 shows HIPPO pathway components with the percentage of amino acids predicted to be in unstructured regions, as calculated by the program IUPred (Dosztanyi et al. 2005a, b). For example, Salvador (WW45), predicted to be 66% disordered, is also efficiently degraded in the ubiquitin-independent pathway (Tsvetkov et al. 2012). Thus, the fact that some of the HIPPO pathway components are unstructured and vulnerable to degradation by default unless they are in a complex, ensures an optimal HIPPO pathway proteostasis.

9.7 From Oncogene to Tumor Suppressor and Back Again

In trying to make sense of complex pathways, we tend to group proteins with similar functions and label them accordingly. Such is the case with "oncogenes" and "tumor suppressors." Tumor suppressors protect cells from becoming cancerous. They prevent unbridled cell division, and will induce apoptosis when DNA damage threatens the integrity of the genome. When proteins are labeled this way, we make predictions about the activity of a protein under a given condition based on its assignment in this context. For example, with DNA damage, we assume tumor suppressors will favor apoptosis. However, this simplification leads to difficulty when the proteins switch roles. Such is the case with YAP, and c-Abl as well. ABL is a well-known oncogene when its N-terminus is fused through translocation, as in the case of chronic myelogenous leukemia (CML), to another protein, such as BCR (Sawyers 1999). In this situation, BCR-ABL is constitutively active, and is cytoplasmic, in contrast to wild-type c-Abl, which has both nuclear and cytoplasmic functions. The altered conformation of BCR-ABL, leading to altered localization, activation, and spectrum of substrates can be used to explain the oncogenicity of BCR-ABL. However, "switches" of wild-type c-Abl activity, causing wild-type c-Abl to be oncogenic, have also been observed. Activation of wild-type c-Abl has been implicated in certain cases of malignant solid tumors of lung and breast (Lin and Arlinghaus 2008). In the case of NSCLC, this may be due to the loss of an endogenous inhibitor of c-Abl, FUS1 (Lin and Arlinghaus 2008). Whether improper activation of c-Abl plays a role in driving other cancers remains to be seen.

As noted above, with DNA damage, c-Abl is active in the nucleus, and phosphorylates YAP, which promotes p73-dependent apoptosis. In this situation, both YAP and c-Abl are tumor suppressors. In a study on breast cancer, YAP was shown to act as a tumor suppressor, and loss of YAP supported tumorigenicity, including increased invasiveness and increased tumor growth in nude mice (Yuan et al. 2008). Nevertheless, numerous mouse genetic studies and analysis of human tumors have shown YAP to be oncogenic (Huang et al. 2005; Overholtzer et al. 2006; Zhao et al. 2008b; Zeng and Hong 2008; Dong et al. 2007). In these models, the pro-proliferative activity of YAP clearly supersedes the pro-apoptotic. The question arises as to why this is true, and which in vivo conditions must prevail in order to support YAP pro-apoptotic activity. From a therapeutic standpoint, a method to switch YAP activity in cancer cells is a lucrative goal. However, in order to accomplish this goal more must be known about YAP regulation in different cell contexts. For example, the upstream HIPPO pathway components appear to have different effects on YAP under different cell contexts. Under high cell density YAP is nuclear excluded through the canonical HIPPO pathway components MST, WW45, and LATS. This prevents YAP coactivation of pro-proliferative genes. In contrast, these HIPPO pathway components, along with RASSF1, are known to promote apoptotic YAP activity in response to DNA damage and other insults (Matallanas et al. 2007). Here, YAP and the other HIPPO pathway proteins are all acting in tumor suppressor mode. The question remains as to what happens at high cell density, when YAP should be

excluded from the nucleus by HIPPO pathway proteins, thereby inhibiting its tumor suppressor activity. Under this condition, does the HIPPO pathway suppress apoptosis? Or, alternatively, is YAP nuclear exclusion suspended upon DNA damage, and if so, what is the mechanism?

Another anti-apoptotic role for YAP was revealed in its ability to compete with LATS for the binding to ASPP1. In this mechanism, LATS promotes the nuclear localization ASPP1 in response to oncogenic stress, and induces ASPP1/p53-driven apoptosis (Aylon et al. 2010). YAP association with LATS in the cytoplasm prevents LATS-mediated ASPP1 translocation. It is still unclear why certain stimuli promote YAP tumor suppressor activity, while others enable the anti-apoptotic activity of YAP. As shown for the case with DNA damage, part of the mechanism could be the status of YAP phosphorylation by c-Abl. However, other mechanisms, such as YAP localization, alternate phosphorylations, or association with different proteins are also likely to affect outcome. For example, c-Abl phosphorylation of ΔNp63 increases its association with YAP and protects from DNA damage-induced apoptosis (Yuan et al. 2010), whereas c-Abl phosphorylation of TAp63 promotes apoptosis (Gonfloni et al. 2009). Phosphorylation of YAP on different sites also regulates its activity. In addition to phosphorylation by LATS (Zhao et al. 2007; Hao et al. 2008; Oka et al. 2008), Akt (Basu et al. 2003), and c-Abl (Levy et al. 2008b), YAP was shown to be multiply phosphorylated on serine and threonine residues in a p38- and JNK-dependent pathway in response to UV and cisplatin (Lee and Yonehara 2012). In addition, YAP activity is modulated by phosphorylation by Src and Yes (Zaidi et al. 2004; Tamm et al. 2011). These multiple modes of regulation enable the multiple functions of YAP. Unfortunately, this complicates our classification of YAP, and prevents easy assignment as either "tumor suppressor" or "oncogene."

9.8 Conclusions and Future Directions

The Hippo pathway, which controls cell fate decisions regarding cell division and apoptosis, must be seen not as a linear route leading from a stimulus on one end, to a defined output at the other. Rather, the Hippo pathway is actually a network, with inputs impinging on the core players coming from different directions, and from different cellular pathways. This complexity means that a given Hippo pathway component's behavior is not fixed; rather, it will be determined by localization, interactions with other proteins, protein level, and posttranslational modifications. Using the case of YAP and the DNA damage response, we see that YAP activity is transformed from pro-proliferative to pro-apoptotic, based on changes in its associations, which is largely regulated by phosphorylation by the non-receptor tyrosine kinase c-Abl. Interestingly, c-Abl regulates several other processes that are highly relevant to Hippo pathway function, including cell proliferation and actin dynamics (Colicelli 2010; Pendergast 2002). It is therefore predicted that c-Abl will be found to play a role in other aspects of Hippo pathway regulation.

The protein–protein interactions that govern activity also contribute to regulation of protein stability. This occurs through protection from ubiquitin-dependent as well as ubiquitin-independent proteasomal degradation. The use of common protein modules for interaction, such as the interaction of WW domains with PY domains, provides for interplay between E3 ubiquitin ligases and their substrates, and competing proteins with complementary protein modules. This scenario was shown for YAP/p73/Itch, and is likely to be a common mechanism for other Hippo pathway components.

Protein–protein interaction also provides a means for escape from ubiquitin-independent degradation, which degrades proteins with unstructured regions. As many regulatory proteins, including those in the Hippo pathway, possess these regions, this mode of degradation/stabilization should be considered when evaluating Hippo regulation.

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Part IV
Cell Cycle and Hippo Signaling

Chapter 10
Hippo in Cell Cycle and Mitosis

Norikazu Yabuta and Hiroshi Nojima

Abstract The Hippo pathway regulates cell growth and cell cycle-dependent processes, including mitosis, cell cycle checkpoints, mitotic checkpoints, and DNA damage response (DDR) checkpoints, thereby preventing the accumulation of abnormal cells with aneuploidy and polyploidy. Moreover, Mst1/2, Lats1/2, Mob1, and Rassf1A primarily colocalize with mitotic regulators, such as Aurora A and Polo, at the centrosome, and then dynamically translocate to the nucleus or the central spindle and the midbody in response to various stimuli. In particular, Lats1/2 play various roles in the DDR checkpoint, maintaining centrosome integrity, mitotic checkpoints (including the spindle assembly checkpoint (SAC)), mitotic exit, cytokinesis, EMT, and cellular senescence. Lats2 also plays a pivotal role in the cell cycle checkpoint via the p53 pathway, thereby functioning as another "guardian" of genome integrity. Therefore, the machinery and related molecules within the Hippo pathway may be potent and promising cancer therapy targets, which may arrest or kill malignant tumor cells without the side effects associated with commonly used treatments.

Keywords Hippo • Cell cycle • Checkpoint • Mitosis • Lats1/2 • Chromosomal instability

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_10,

10.1 Introduction

Human malignant cancer cells exhibit two major hallmarks: abnormal cell proliferation and chromosomal instability (CIN) (Hanahan and Weinberg 2011; Gordon et al. 2012). The main causes of CIN are aberrant regulation of cell cycle checkpoints and abnormal mitosis, both of which lead to the accumulation of genetic errors and to the loss or gain of genetic material, which are then passed on to the next generation. In particular, malfunction of mitotic progression or the spindle assembly checkpoint (SAC) results in centrosome hyperamplification, defective centrosome separation, and incorrect microtubule-kinetochore attachments. This leads to chromosome missegregation or failure of cytokinesis, followed by the generation of aneuploid cells and genetically unstable tetraploid cells, which facilitate tumor progression (Storchova and Kuffer 2008; Davoli and de Lange 2011; Vitale et al. 2011). Therefore, the cellular machinery is stringently organized by the actions of various molecules: cell cycle and checkpoint regulators, such as Cyclins/Cdks, Cdk inhibitors, pRb, E2F, Cdc25, and p53; DNA damage checkpoints, such as ATM (Ataxia-Telangiectasia muted), ATR (Ataxia-Telangiectasia and Rad-3-related), Chk1, and Chk2; mitotic kinases, such as Cdk1, Aurora, Polo, NIMA, and BubR1; and apoptosis regulators, such as Bax, Bcl, and caspases.

The Hippo signaling pathway, which is highly conserved in fruit flies and higher eukaryotes, regulates organ size by controlling cell proliferation and apoptosis, and plays an essential role in the suppression of tumor cell growth and the self-renewal of stem cells (Pan 2010; Zhao et al. 2011). In mammalian cells, four kinds of serine (S)/threonine (T) kinases, Mst1 (mammalian sterile 20-like kinase 1), Mst2, Lats1 (large tumor suppressor 1), and Lats2; two kinds of adaptor protein, hWW45/hSav1 and Mob1 (*M*ps *o*ne-*b*inder 1); and two kinds of the transcriptional coactivator, Yap (yes-associated protein) and Taz (transcriptional coactivator with PDZ-binding motif) form the core components of the canonical Hippo pathway. Mst1/2, Lats1/2, WW45, Mob1, and Yap/Taz are functionally equivalent to their fruit fly homologs, Hpo (Hippo), Wts (Warts/Lats), Mats (mob as tumor suppressor) and Yki (Yorkie), respectively (see Fig. 10.5, left panel).

These core components comprise the main phosphorylation signaling cascade in response to upstream activation signals, such as cell–cell contact. In human cells, activated Mst1/2 kinases, along with their activator, WW45, cooperatively phosphorylate both Lats1/2 kinases (at S909* and T1079 at the C-terminus of Lats1, and at S872* and T1041 at the C-terminus of Lats2; *these sites possibly function as autophosphorylation sites) and their regulator, Mob1 (at T12 and T35), thereby inducing formation of activated Lats1-Mob1 and/or Lats2-Mob1 complexes. This results in phosphorylation of Yap/Taz (at S127 and S381 in Yap, and at S89 and S311 in Taz), preventing their nuclear translocation and the subsequent induction of cell-proliferative and anti-apoptotic gene expression (Pan 2010; Zhao et al. 2011). Phosphorylation of Yap-S127 sequesters Yap within the cytoplasm via binding to 14-3-3 proteins, whereas phosphorylation of Yap-S381 targets Yap for degradation via ubiquitin-mediated proteolysis (Zhao et al. 2007, 2010). Moreover, the Hippo core pathway engages a variety of upstream regulators and modulators, such as Fat1/4, Merlin/

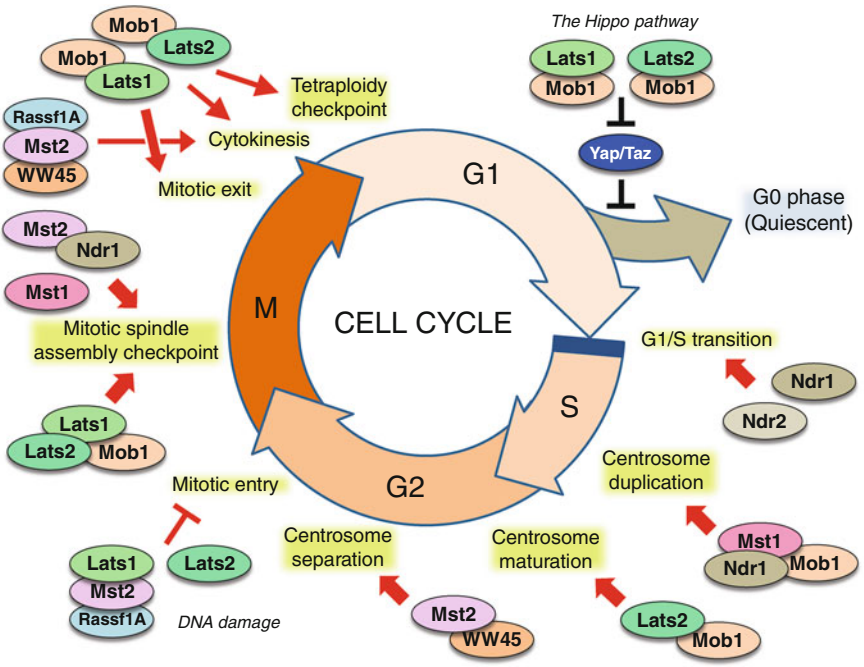

Fig. 10.1 Roles of the different Hippo pathway components in cell cycle regulation. The core Hippo pathway inhibits the function of Yap/Taz and increases the expression of genes involved in cell proliferation. In other words, the core Hippo components induce growth arrest via the canonical Hippo pathway, whereas they play pivotal roles in controlling the cell cycle and cell cycle checkpoints. Because Kibra is phosphorylated by Aurora A, and contributes to Lats2 stabilization, Kibra may regulate Aurora A/Lats2-mediated cell cycle functions (not shown here)

Nf2, Kibra, Tao-1/TAOK, Willin/FRMD6, Rassf, Ajuba (a fruit fly homolog of dJub), Itch, and Angiomotin/Amot (Zhao et al. 2011; Boggiano and Fehon 2012).

The Hippo pathway is an important piece of cellular machinery that induces cell growth arrest in response to cell–cell contact (known as "contact inhibition"), which is thought to function during oncogenic suppression. Studies of human tumor samples show that many regulators of the Hippo pathway appear to be dysfunctional; for example, Fat1/4, Merlin/Nf2, Kibra, Mst1/2, and Lats1/2 are downregulated, whereas Yap/Taz are overexpressed (Pan 2010). This suggests that the Hippo pathway plays a crucial role in the tumor suppressor pathways in human malignant cancers. However, it is unclear how the Hippo pathway is involved in suppressing and eliminating malignant tumor cells showing aneuploidy or polyploidy.

Recently, some components of the Hippo pathway were found to regulate CIN by interacting with the cell cycle checkpoint machinery and through mitotic regulation (Fig. 10.1). This chapter provides an overview of the current knowledge about the role played by the Hippo pathway in regulating the cell cycle and mitosis, with particular focus on the function of Lats1/2 kinases and their regulators as central players in the cell cycle, mitosis, and checkpoint regulation.

10.2 Mst1 and Mst2

10.2.1 Mst1 and Mst2 as Members of the MEN/SIN Family

The mammalian Hippo homologs, Mst1 and Mst2, belong to the sterile 20-like kinase family, and have some biological functions that are independent of the canonical Hippo pathway (namely Yap/Taz-independent growth control). For example, they are involved in stress-induced apoptosis via the cleavage and subsequent nuclear translocation of their proteins (Ling et al. 2008; Matallanas et al. 2008). Of note, recent reports show that both Mst1 and Mst2 play important roles in both mitosis and apoptosis by taking advantage of some Hippo pathway components; this function appears to be evolutionarily conserved from yeast to humans (Bardin and Amon 2001; Hergovich and Hemmings 2012). Both Cdc15 in budding yeast and Sid1 in fission yeast, which are functional counterparts of Mst1/2, localize to the nascent centrosome (the so-called spindle pole body [SPB] in yeast) at anaphase, and to the contractile ring (the so-called bud neck and septum site in budding and fission yeast, respectively) at cytokinesis (Guertin et al. 2000; Menssen et al. 2001). At the onset of late mitosis, activated Cdc15 and Sid1 kinases directly phosphorylate and activate the Dbf2/Mob1 and Sid2/Mob1 kinase complexes (Lats1/2-Mob1 complexes in mammals), which in turn inhibit mitotic CDK1 through the release of Cdc14 phosphatase (budding yeast Cdc14 and fission yeast Clp1) from the nucleolus into the nucleoplasm and cytoplasm. Eventually, these signaling cascades, which are known as the mitotic exit network (MEN) in budding yeast and the septation initiation network (SIN) in fission yeast, allow the cells to exit mitosis, complete cytokinesis, and then execute a successful transition from mitosis into the next G1 phase (Bardin and Amon 2001). On the other hand, there are no reports that *hippo* mutant fruit flies show a failure of cytokinesis, although RNAi-mediated *hippo*-knockdown cells do show severe defects in mitotic spindle and central spindle formation (Bettencourt-Dias et al. 2004).

10.2.2 Centrosome Regulation by Mst1 and Mst2

Like Cdc15/Sid1 in yeast, mammalian Mst2 localizes to the centrosomes during interphase, at the spindle poles during mitosis, and at the midbody (the final convergent structure of the contractile ring) during cytokinesis (Guo et al. 2007). Moreover, Mst1/2 kinases are activated by increasing protein levels and kinase activity in mitotic cells treated with nocodazole, a molecule that depolymerizes the microtubules (Praskova et al. 2008). In cells arrested at M phase by treatment with nocodazole, Mst1 and Mst2 are phosphorylated on the activation loop at threonine 183 (T183) and T180, respectively; activated Mst2 then phosphorylates both Lats1 and Mob1 to activate the Lats1/Mob1 complex (Praskova et al. 2008). Interestingly,

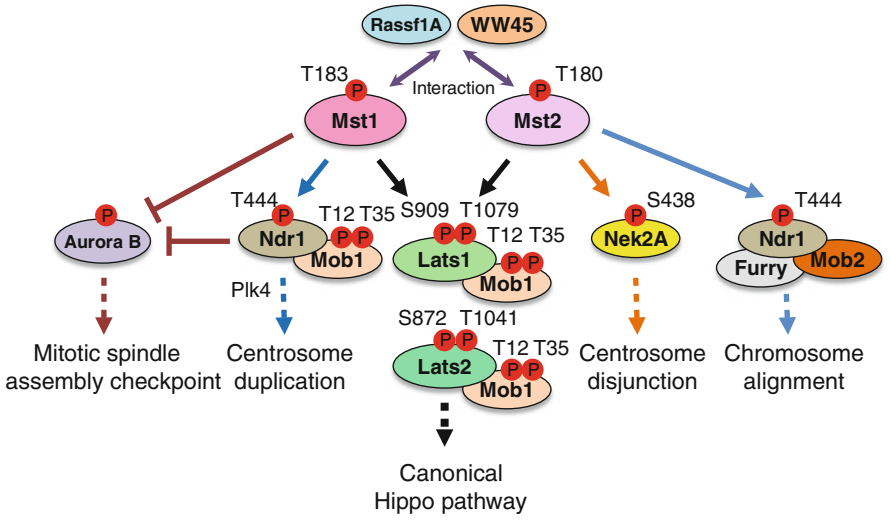

Fig. 10.2 Mst1/2 kinases regulate the Hippo pathway and the cell cycle by phosphorylating different downstream targets. Mst1/2 are activated by binding to Rassf1A and/or WW45. Five axes (the Mst1/2-Lats1/2 axis, the Mst1-Aurora B axis, the Mst1-Ndr1 axis, the Mst2-Nek2A axis, and the Mst2-Ndr1 axis) regulate five subcellular events: the canonical Hippo pathway, the mitotic spindle assembly checkpoint, Plk4-driven centrosome duplication, centrosome disjunction, and chromosome alignment, respectively

association of Mst1/2 to two functional scaffold proteins, RASSF1A and WW45 (also known as hSav1 [a Salvador homolog] in mammals) within the Hippo pathway is initially required for mitotic activation of the Mst1/2 kinases, which takes place at the centrosome (Guo et al. 2007). Moreover, Mst2 promotes the association between RASSF1A and WW45 via their C-terminal coiled-coil motifs, known as Sav/Rassf/Hpo (SARAH) domains (Guo et al. 2007). Thus, it is suggested that Mst1/2 is efficiently activated in response to mitotic signaling by a positive feedback loop between Mst1/2 and RASSF1A/WW45 on the centrosome.

Indeed, Mst1 and Mst2 appear to play key roles in centrosomal integrity. Mst1, together with Mob1, regulates centrosome duplication (Hergovich et al. 2009). SiRNA-mediated downregulation of Mst1 or Mob1 inhibits centriole duplication in human osteosarcoma U2OS cells. Moreover, Mst1 directly phosphorylates Ndr1 kinase (the second member of the Dbf2 kinase family, which is equivalent to Tricornered [Trc] in fruit flies) at T444 on the hydrophobic motif to activate the Ndr1/Mob1 complex. This is essential for Polo-like kinase 4 (Plk4)-driven centriole duplication via localization of the distal end-capping protein, CP110, to the centriole, but not that of the cartwheel protein HsSAS-6 (Habedanck et al. 2005). Notably, the other Hippo pathway components, WW45 and RASSF1A, appear to be dispensable for centrosome duplication mediated by the Mst1-Ndr1-Mob1 pathway (Hergovich et al. 2009) (Fig. 10.2).

On the other hand, Mst2 plays a pivotal role in centrosome disjunction. Specifically, Mst2 and WW45 directly interact with the NIMA-related kinase Nek2A (but not with Nek2B) via the SARAH domains in their C-terminal regions, and recruit it to the centrosome (Mardin et al. 2010) (Fig. 10.2). Moreover, Mst2 phosphorylates Nek2A at S438, which promotes the recruitment of Nek2A to the centrosome, but does not increase its kinase activity. Simultaneously, the Mst2-WW45 complex contributes to the phosphorylation of two centrosomal linker proteins, c-Nap1 and Rootletin (both major Nek2A phosphorylation targets, which bridge the gap between the two centrosomes). Interestingly, other Hippo pathway components, such as Lats1/2, Rassf1A, and Yap, are dispensable for the centrosomal targeting of Nek2A. Downregulation of Mst1/2, as well as Nek2 and WW45, prevents centrosome splitting during late G2 phase, which can be rescued by overexpression of a constitutively phosphorylated Nek2A mutant (Nek2A-4D, harboring a S438D mutation). Furthermore, Mst2 is directly phosphorylated by Polo-like kinase 1 (Plk1), which prevents the association between Mst2-Nek2A and protein phosphatase 1γ (PP1γ), thereby promoting Nek2A kinase activity (Mardin et al. 2011). Therefore, Mst2 and WW45 are key regulators of centrosome disjunction downstream of Plk1 via Nek2A-induced c-Nap1/Rootletin dissociation and subsequent bipolar spindle formation. In support of this, evidence from budding yeast shows that a Polo-like kinase homolog, Cdc5, is required for the recruitment of Cdc15 (Mst1/2) onto the SPB (Rock and Amon 2011).

10.2.3 Chromosome Regulation by Mst1 and Mst2

Importantly, siRNA-mediated downregulation of Mst1 frequently causes chromosome misalignment and anaphase delay in human cervical cancer HeLa cells through Mad2- and BubR1-dependent spindle checkpoint activation. In particular, Mst1 associates with, and directly phosphorylates, Aurora B, a mitotic spindle checkpoint kinase. This phosphorylation negatively modulates Aurora B kinase activity and stabilizes the kinetochore-microtubule attachments (Oh et al. 2010). Like Mst1 depletion, downregulation of Mst2 causes chromosome misalignment, although it is unclear whether Mst2 directly phosphorylates Aurora B and/or regulates its activity (Oh et al. 2010). Instead, Mst2 contributes to the mitotic activation of Ndr1 kinase in cooperation with its activator, Mob2, and a scaffold protein called Furry (Fry) (present in the spindle microtubules), thereby regulating the precise alignment of mitotic chromosomes (Chiba et al. 2009).

Taken together, it is likely that mammalian Mst1 and Mst2 are essential regulators of centrosomal integrity (Mst1 regulates centrosome duplication, whereas Mst2 regulates centrosome disjunction), and of accurate chromosome alignment and segregation during mitosis. However, although yeast homologs Cdc15/Sid1 are mainly involved in mitotic exit or cytokinesis, the precise role of Mst1/2 during cytokinesis is not fully understood (apart from its ability to restore cytokinesis in Rassf1A-deficient mouse embryo fibroblasts (Guo et al. 2007)).

10.3 Lats1 and Lats2

10.3.1 Protein Structures of Lats1/2

Lats1 and Lats2 (Lats1/2) are mammalian homologs of yeast mitotic kinases: Dbf2/Dbf20 in budding yeast and Sid2 in fission yeast (Tao et al. 1999; Yabuta et al. 2000). Lats1 and Lats2 are also known as Warts/h-warts and Kpm, respectively (Nishiyama et al. 1999; Hori et al. 2000). Compared with the primary structures of their yeast homologs, Lats kinases in higher eukaryotes, including fruit fly Warts/Lats, nematode Lats1, and mammalian Lats1/2, possess a long, stretched N-terminal non-kinase region (approximately 700 amino acids) upstream of the highly conserved serine/threonine kinase domain (the kinase activity of which is regulated by Mst1/2). However, this region is not present in yeast Dbf2/Sid2 and mammalian Ndr1/2 (Visser and Yang 2010). Because organ size and tumor formation are not a concern for unicellular organisms such as yeast, the N-terminal regions of Lats kinases in higher eukaryotes may play various roles in tumor development and malignancy, not only through the canonical Hippo pathway but also through the other mechanisms, such as cell cycle and checkpoint controls. Moreover, a comparison between the N-terminal halves of mammalian Lats1 and Lats2 reveals that they share low sequence similarity, apart from Lats conserved domain 1 (LCD1) and LCD2, which may be functionally important for tumor suppression. By contrast, their C-terminal kinase domains are highly conserved (85% and 80% sequence identity between human and mouse, respectively) (Li et al. 2003). It is suggested that Lats1 and Lats2 possess both common and mutually exclusive physiological functions.

Mammalian Lats1/2 kinases are involved in cell cycle regulation and malignancy (Fig. 10.1). Although the N-terminal regions of Lats1/2 interact with some Hippo pathway regulators, such as Mob1, Yap, Taz, and Kibra (Pan 2010; Sudol and Harvey 2010; Visser and Yang 2010), they also interact with various cell cycle regulators. For example, the N-terminus of Lats1 physically interacts with the mitotic kinase, Cdc2 (Cdk1), the actin filament assembly factor, Zyxin, and a regulator of actin dynamics, LIM kinase 1 (LIMK1) (Tao et al. 1999; Hirota et al. 2000; Yang et al. 2004), whereas the N-terminus of Lats2 interacts with centrosomal proteins such the LIM protein, Ajuba, and Aurora A kinase (Abe et al. 2006; Toji et al. 2004). Moreover, a variety of protein kinases phosphorylate the N-terminal non-kinase regions of Lats1/2, although their C-termini are phosphorylated by Mst1/2 kinases (S909 and T1079 of Lats1, and S872 and T1041 of Lats2) (Fig. 10.5). The N-terminal region of Lats1 is phosphorylated by Cdc2/cyclin B (at S613), NUAK1 (at S464), and PKCδ (at S464), whereas that of Lats2 is phosphorylated by Aurora A (at S83 and S380) and Chk1/2 (at S408); these phosphorylations regulate their subcellular localization, protein stability, and/or enzymatic activity (Morisaki et al. 2002; Chan et al. 2005; Takahashi et al. 2006; Humbert et al. 2010; Okada et al. 2011; Yabuta et al. 2011).

10.3.2 Lats1/2 Are Human Tumor Suppressors

The *LATS1/2* genes are candidate human tumor suppressor genes. Human *LATS2* maps to the chromosome 13q11-q12 region, in which loss of heterozygosity has been frequently observed in various primary cancers (Yabuta et al. 2000). In fact, expression of *LATS1/2* is downregulated in a variety of human malignant tumors, such as aggressive breast cancers, acute lymphoblastic leukemia, non-small cell lung cancer, and astrocytoma, by hypermethylation of their promoter regions (Takahashi et al. 2005; Jiménez-Velasco et al. 2005; Jiang et al. 2006). In human breast and prostate cancer samples, *LATS2* expression is downregulated by a defect in the transcriptional factor, FOXP3 (Li et al. 2011), and inactivating mutations within the *LATS2* coding region have also been reported in human malignant mesothelioma (Murakami et al. 2011). In particular, expression of *LATS2* in human testicular germ cell tumors, colorectal cancer, gastric cancer cell lines, and esophageal cancer cell lines is downregulated by micro (mi)RNAs (miR-372 and/or miR-373) (Voorhoeve et al. 2006; Cho et al. 2009; Lee et al. 2009; Yamashita et al. 2012). Moreover, Lats1 knockout (*Lats1*$^{-/-}$) mice are susceptible to soft-tissue sarcomas and ovarian tumors, although Lats2 knockout (*Lats2*$^{-/-}$) mice are embryonic lethal before embryonic day 12 (St. John et al. 1999; McPherson et al. 2004; Yabuta et al. 2007). Notably, disruption of *Lats2* in MEFs (mouse embryonic fibroblasts) causes centrosomal fragmentation, abnormal mitotic spindle formation, chromosomal missegregation, and failure of cytokinesis, thereby inducing the CIN that is a hallmark of malignant tumor cells (McPherson et al. 2004; Yabuta et al. 2007). Taken together, these reports suggest that Lats1 and Lats2 cooperatively regulate the cell cycle and tumor suppression in mammalian cells.

10.3.3 Lats1/2 Play Pivotal Roles in the Cell Cycle Checkpoint

Lats1/2 play important roles in cell growth and the regulation of organ size through the Hippo pathway, and have a role in chromosomal stability through cell cycle checkpoint regulation. Specifically, enforced expression of Lats1 in MEFs and human cancer cells harboring a nonfunctional p53 tumor suppressor (such as HeLa and C33A cells) causes G2/M arrest via the binding and inactivation of Cdc2 kinase, whereas it triggers apoptosis in human cancer cells harboring wild-type p53 (such as A549 and HCT116 cells) via induction of p53 or proapoptotic Bax (Tao et al. 1999; Yang et al. 2001; Xia et al. 2002). By contrast, exogenous expression of Lats2 in mouse NIH3T3/*v-ras* cells causes G1/S arrest by inactivating Cdk2/Cyclin E (which express wild-type p53), but causes G2/M arrest in functional p53-deficient HeLa cells by inactivating Cdc2/Cyclin B, leading to apoptosis (Kamikubo et al. 2003; Li et al. 2003). In lung cancer cells such as A549 and H1299, overexpression of Lats2 causes apoptosis by downregulating expression of the anti-apoptotic proteins, Bcl-2 and Bcl-X_L, a mechanism that is independent of p53 status (Ke et al. 2004).

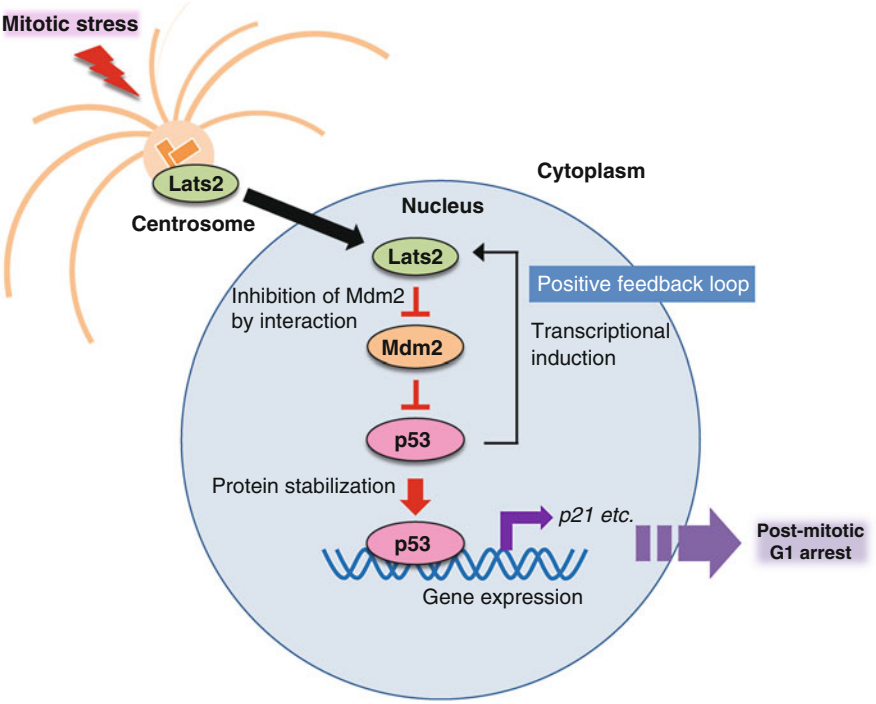

Fig. 10.3 The Lats2-Mdm2-p53 pathway regulates the G1 tetraploidy checkpoint. In response to mitotic stress, the Lats2-Msm2-p53 axis forms a positive feedback loop and subsequently promotes post-mitotic G1 arrest by inducing the expression of *p21* (see text)

The p53 tumor suppressor is a key regulator of the cell cycle checkpoint that activates and functions as a transcription factor to induce cell cycle arrest and apoptosis in response to various stimuli and stresses (Aylon and Oren 2011). Importantly, Oren's group showed that Lats2 positively regulates p53 at the cell cycle checkpoint via its involvement in a novel tumor suppressor axis, Lats2-Mdm2-p53 (Aylon et al. 2006) (Fig. 10.3). When cells are exposed to microtubule poisons, such as nocodazole, which elicit mitotic stress in cells, Lats2 translocates from the centrosome to the nucleus, where it accumulates. Nuclear Lats2 directly binds to and inhibits the E3 ubiquitin ligase, Mdm2, thereby stabilizing and activating the p53 protein; p53 then selectively binds to the promoter region of *LATS2,* rapidly upregulating Lats2 expression via a positive feedback loop. As a result, an abundance of p53 upregulates expression of the Cdk inhibitor, p21, thereby inducing G1 arrest of aberrant cells that exit mitosis without proper sister chromatid segregation or cytokinesis (mitotic slippage), and preventing tetraploidization (the G1 tetraploidy checkpoint) (Aylon et al. 2006). Moreover, an E3 ubiquitin ligase, Fbw7, also cooperatively regulates p53-dependent induction of Lats2 and p21 expression by degrading Cyclin E and Aurora A (Finkin et al. 2008).

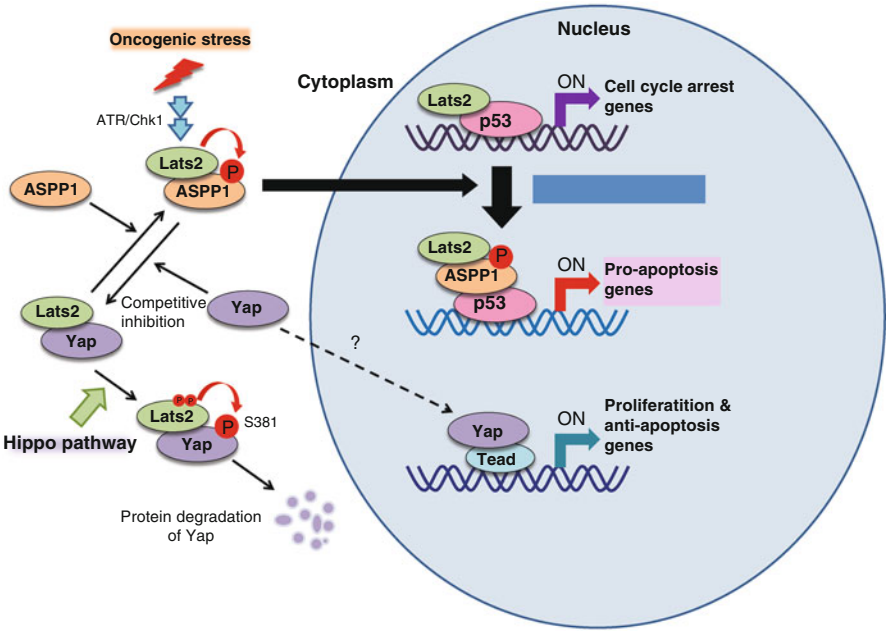

Fig. 10.4 The Lats2-ASPP1-p53 pathway regulates apoptosis. In response to oncogenic stress, probably through ATR-Chk1 signaling, the Lats2-ASPP1-p53 axis promotes the translocation of p53 from the promoters of genes involved in cell cycle arrest, such as *p21,* to the promoters of genes associated with apoptosis, such as *BAX* and *PIG3* (see text). This pathway is competitively inhibited by Yap proteins, which may be inhibited by the activated Hippo pathway

More importantly, the nuclear accumulation and translocation of Lats2 and subsequent p53 activation are also increased in response to oncogenic H-Ras via the ATR-Chk1 kinase-mediated stress checkpoint pathway (see below), which induces apoptosis or cellular senescence in polyploidy cells by quenching H-Ras-induced transformation and tumor progression (Aylon et al. 2009). So how does Lats2 efficiently promote apoptosis of transformed cells despite them being highly oncogenic? In response to oncogenic stress, Lats2 associates with, and directly phosphorylates, apoptosis-stimulating protein of p53-1 (ASPP1), which diverts p53 from the promoters of cell cycle genes such as *p21* to the promoters of proapoptotic genes such as *BAX* and *PIG3* (Aylon et al. 2010). Thus, depletion of Lats2 or ASPP1 by siRNA fails to trigger apoptosis in transformed cells, thereby increasing the number of polyploid cells. This suggests that Lats2 plays a pivotal role in the prevention of polyploidy and in the suppression of tumor malignancy. Notably, a Hippo pathway effector, Yap, competitively inhibits the interaction between Lats2 and ASPP1 and the subsequent induction of apoptosis by directly interacting with Lats2 (Aylon et al. 2010) (Fig. 10.4). Lats1 may also competitively interact with the ASPP1 or Yap proteins (Vigneron et al. 2010). Overexpression of kinase-inactivated Lats1 in Rat1 fibroblasts leads to mitotic slippage and the accumulation of tetraploidy in a

dominant-negative manner, which is accompanied by the downregulation of p53 (Iida et al. 2004). Interestingly, Lats1 can also directly interact with, and inhibit, the ability of Mdm2 to destabilize the p53 protein, thereby inducing apoptosis in colorectal cancers with a K-Ras mutation (Matallanas et al. 2011). However, it is likely that Lats1 enforces these checkpoint programs downstream of the activated Rassf1A-Mst2 pathway, whereas Lats2 takes advantage of the ATR-Chk1 pathway (Aylon et al. 2009; Matallanas et al. 2011).

Therefore, Lats1 and Lats2 are key regulators of the p53-mediated cell cycle checkpoint, which maintains the correct chromosome number through post-mitotic G1 arrest (the G1 tetraploidy checkpoint) and apoptosis. Consistent with this, fruit fly Dmp53 (the fruit fly homolog of p53)-induced apoptosis is reduced in *warts* mutants and in *hippo* and *salvador* mutants (Colombani et al. 2006).

10.3.4 Lats1/2 Also Regulate the DNA Damage Checkpoint

The DNA damage response (DDR) is crucial for maintaining genomic stability, and mainly functions through two DNA damage signaling pathways: the ATM and ATR pathways (Bartek et al. 2007; Ciccia and Elledge 2010). ATR kinase regulates the downstream effector kinase, Chk1, in response to DNA damage caused by single-strand breaks (SSBs) or stalled replication forks (referred to as the ATR-Chk1 pathway). Since Lats2 functions downstream of the ATR-Chk1 pathway in response to H-RasV12-induced oncogenic stress (Aylon et al. 2009), Lats2 may also play a role in regulating the DNA damage checkpoint.

Indeed, the authors found that Lats2 is directly phosphorylated (at S408 at its N-terminus) by Chk1 kinase in response to UV irradiation; activated Lats2 then directly phosphorylates 14-3-3 proteins at S59 (Okada et al. 2011). Interestingly, Lats2-phosphorylated 14-3-3 proteins accumulate in cytoplasmic foci known as P (processing)-bodies, in which mRNA degradation, translational repression, and mRNA surveillance are stringently regulated by miRNAs and scaffold proteins such as GW182. The accumulation of 14-3-3 proteins within the P-body, and enlargement of the P-body itself, are induced by UV damage and are dependent on Lats2, but not Lats1 (Okada et al. 2011). Thus, it is likely that the Chk1-Lats2-P-body pathway efficiently represses translation after UV damage (known as the "CLP pathway") (Fig. 10.5, middle).

However, in fruit flies, DNA damage caused by double-strand breaks (DSBs) enhances the activity of Hippo kinase in a Dmp53-dependent manner (Colombani et al. 2006). In mammals, Lats1 and Mst2 are activated downstream of Rassf1A, which is phosphorylated at S131 by ATM after DNA damage caused by DSBs, thereby inducing the stabilization of the proapoptotic transcriptional factor, p73, and subsequently triggering apoptosis (Hamilton et al. 2009). Moreover, Lats1 phosphorylates S445 of MYPT1 (myosin phosphatase-targeting subunit 1), which promotes the dephosphorylation of Plk1 by PP1C after DSBs, which results in the

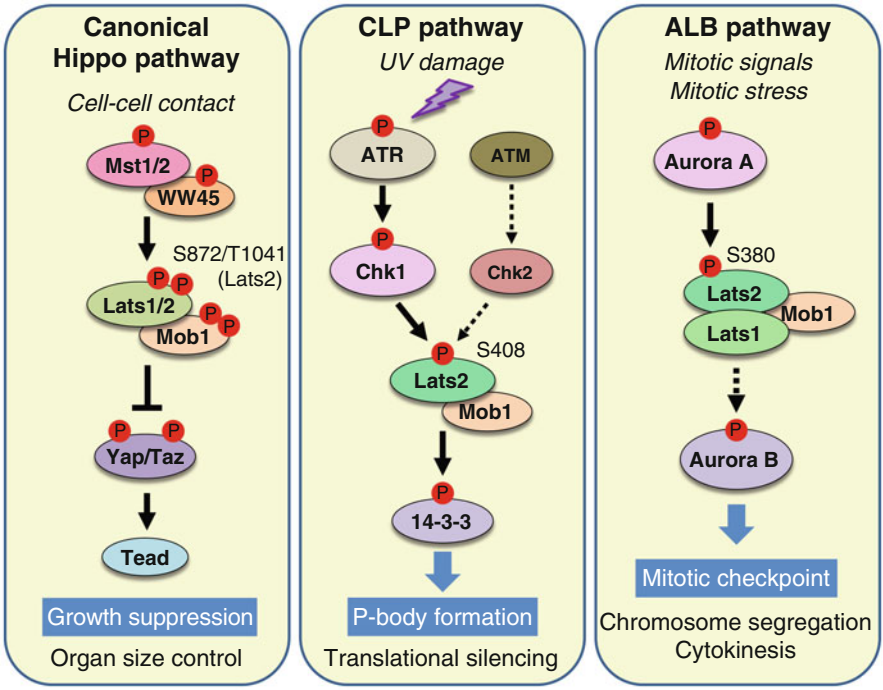

Fig. 10.5 Novel Lats2-mediated signaling pathways. The canonical Hippo pathway regulates growth suppression to control organ size by inhibiting the Yap/Taz-Tead transcriptional system (left), whereas the Chk1/2-Lats2-P-body axis (CLP pathway, middle) and Aurora A-Lats1/2-Aurora B axis (ALB pathway, right) regulate P-body formation in response to UV damage and the mitotic checkpoint, respectively

suppression of Plk1 activity during G2/M transition (Chiyoda et al. 2012). It remains unclear whether Lats2 participates in these pathways after DSBs. Future studies are needed to understand the detailed molecular mechanisms underlying the Lats1/2-mediated pathways involved in the DDR.

10.3.5 Lats1/2 Control Mitotic Progression and Cytokinesis

Dbf2, a Lats/Warts homolog in budding yeast colocalizes with its coactivator, Mob1, and the upstream kinase, Cdc15, at the SPB (or the centrosome in mammals) during anaphase. A fraction of these proteins then translocates to the bud neck during cytokinesis, where they promote MEN and cytokinesis in response to Cdc15 activation (Frenz et al. 2000; Hwa Lim et al. 2003). Another yeast counterpart, Dbf20, probably plays a minor role in the MEN (Toyn and Johnston 1994). The Lats homolog, Sid2, in fission yeast also colocalizes with Mob1 at the SPB throughout

mitosis and at the septum site during cytokinesis, where it forms an essential component of the SIN to regulate cytokinesis (Sparks et al. 1999; Hou et al. 2004). Therefore, mammalian Lats1/2 may also localize to the mitotic apparatus and actually regulate mitotic progression, mitotic exit, and cytokinesis.

In human cancer cells, Lats2 normally localizes to the centrosome, cytoplasm, and nucleus during interphase (Toji et al. 2004). *Lats2*$^{-/-}$ MEFs show amplification of pericentriolar material (PCM) such as γ-tubulin, but not of the centrioles (Yabuta et al. 2007). Moreover, siRNA-mediated depletion of Lats2 in HeLa cells also leads to loss of γ-tubulin accumulation at the centrosome and disintegration of spindle microtubules (Abe et al. 2006). These results suggest that Lats2 regulates centrosomal integrity, including centrosome fragmentation, but probably not centriole duplication. Although Lats1 also localizes to the centrosome (Nishiyama et al. 1999), there is no report to date that knockout or knockdown of Lats1 causes centrosomal abnormalities in mammalian cells. Alternatively, overexpression of the kinase-inactive form of Lats1 or Lats2 in U2OS cells seems to have no effect on centrosomal duplication, whereas overexpression of wild-type Ndr1, but not kinase dead Ndr1, leads to centrosomal overduplication, probably through the Mst1-Ndr1-Mob1 pathway (Hergovich et al. 2007, 2009). Interestingly, Lats2 associates with centrosomal proteins such as Aurora A and its activator, Ajuba (Toji et al. 2004; Abe et al. 2006). Similarly to Lats2 depletion, knockdown of Ajuba results in disintegration of the centrosome and mitotic spindle. Moreover, Ajuba physically interacts with Lats1/2, which prevents Lats1/2-mediated phosphorylation of YAP in the Hippo pathway (Das Thakur et al. 2010). Thus, the Lats1/2-Ajuba-Aurora A complex may cooperatively regulate centrosome maturation and spindle organization on the centrosome.

While studying the role of mammalian Lats2 in cell cycle regulation, the authors found that human Lats2 is multiply phosphorylated during cell cycle progression, including both at interphase and during mitosis (Toji et al. 2004). Like Lats2, Lats1 is also phosphorylated during mitosis (Tao et al. 1999; Nishiyama et al. 1999; Hori et al. 2000; Toji et al. 2004). These results suggest that Lats1/2 are stringently regulated by the Hippo pathway and by the cell cycle machinery, including the mitotic checkpoint and cytokinesis. In fact, the N-terminus of Lats2 is phosphorylated at multiple sites (at least two: S83 and S380) by Aurora A kinase during mitosis (Toji et al. 2004; Yabuta et al. 2011). Moreover, Lats2 colocalizes with Aurora A at the centrosome during mitosis (Toji et al. 2004). Aurora A is a member of the Aurora kinase family, which regulates centrosome integrity and cell division by phosphorylating numerous proteins that play a critical role in mitosis (Nigg 2001; Salaun et al. 2008). Interestingly, the subcellular localizations of phosphorylated Lats2 are different during the mitotic phase (Yabuta et al. 2011): Lats2 phosphorylated on S83 localizes to the nucleus during early mitosis and at the centrosome throughout mitosis, whereas S380-phosphorylated Lats2 localizes to the nucleus during early mitosis and to the chromosomes during metaphase. It then moves to the central spindle/spindle mid-zone during anaphase/telophase. During cytokinesis, phosphorylated Lats2 is concentrated at the midbody. It is suggested that Aurora A regulates the mitotic localization of Lats2 via different patterns of phosphorylation. On the other hand, Lats1 also localizes at the mitotic apparatus

(centrosome, mitotic spindle, central spindle, and midbody), where it associates with Zyxin to enable mitotic progression (Hirota et al. 2000). Although Lats1 may contain putative Aurora A phosphorylation sites (Sardon et al. 2010), it is unclear whether Lats1 is actually phosphorylated by Aurora A. Instead, Lats1 interacts with Cdc2 (Tao et al. 1999) and is phosphorylated at S613 by the Cdc2/Cyclin B complex during mitosis; however, the role of Cdc2/cyclin B-phosphorylated Lats1 is not clear (Morisaki et al. 2002).

Aurora B, another member of the Aurora kinase family, forms the chromosomal passenger complex (CPC) by interacting with the inner centromere protein (INCENP), Survivin, and Borealin/Dasra-B, enabling proper kinetochore-microtubule attachments, chromatid cohesion, spindle formation through the SAC, and cytokinesis (Ruchaud et al. 2007). Of note, a fraction of the S380-phosphorylated Lats2 in HeLa and U2OS cells colocalizes with Aurora B at the chromosome arms, the central spindle, and the midbody (Yabuta et al. 2011). Moreover, Lats2 physically interacts with Aurora B, as well as Aurora A. Interestingly, Lats2 can also interact with Lats1 in the presence of Mob1A, and Lats1 kinase plays a role in the phosphorylation of Aurora B. Indeed, the expression of a non-phosphorylated Lats2 mutant (S380A) in HeLa cells promotes lagging chromosomes, chromosome bridges, aberrant nuclear formation, micronuclear formation, and multinucleation, which are similar to the phenotypes induced by abnormal regulation of Aurora B (chromosome missegregation and cytokinesis failure). Consistent with this, Rat-1 cells overexpressing the kinase-inactive form of Lats1 show mitotic delay and SAC activation (Iida et al. 2004). Therefore, it is probable that the Aurora A-Lats1/2-Aurora B axis functions as a novel pathway to ensure accurate mitotic progression (known as the "ALB pathway") (Fig. 10.5, right). This pathway is partially conserved in budding yeast; namely yeast Mob1 (a cofactor and activator of Dbf2/Lats) allows the single yeast Aurora homolog, Ipl1p, to dissociate from the kinetochore region, and maintains the localization of CPC-containing Ipl1, Bir1 (Survivin), and Sli15 (INCENP) on the anaphase spindle (Stoepel et al. 2005). Since dysfunction of the ALB pathway promotes mitotic delay and chromosome missegregation (Yabuta et al. 2011), prolonged mitosis may be a trigger for the subsequent G1 tetraploidy checkpoint- or mitotic catastrophe-mediated cell death seen in mammalian cells expressing fully functional p53 (Vitale et al. 2011).

Lats1/2 proteins are concentrated, along with their activator Mob1A, at the midbody during cytokinesis (Bothos et al. 2005). *Lats2*$^{-/-}$ MEFs frequently show failure of cytokinesis (approximately 20–30% of primary MEFs), thereby giving rise to multinucleated cells (Yabuta et al. 2007), whereas *Lats1*$^{-/-}$ cells give rise to low numbers of multinucleated cells (approximately 5–6% of MEFs or brain cortex cells) (Yang et al. 2004). By contrast, even if wild-type Lats1 is ectopically overexpressed in human cancer cells such as HeLa, it does not affect the completion of cytokinesis. Nevertheless, Lats1 directly interacts with LIMK1, and inhibits LIMK1 kinase activity toward the actin-depolymerizing protein Cofilin, thereby preventing LIMK1-induced cytokinesis defects. Moreover, the kinase activity of Lats1 is not essential for the inhibition of LIMK1 (Yang et al. 2004). Since Lats1 knockdown, as well as Mob1A knockdown, in U2OS cells results in a prolonged telophase,

the kinase activity of Lats1 may act as a mammalian form of MEN kinase to promote exit from mitosis (Bothos et al. 2005). On the other hand, some of the *Lats2*$^{-/-}$ MEFs fail to complete cytokinesis, whereas others show accelerated exit from mitosis along with the premature downregulation of mitotic regulators (Yabuta et al. 2007). Thus, Lats2, unlike Lats1, may regulate cytokinesis by acting more like a mammalian SIN kinase than a MEN kinase.

10.3.6 Other Roles of Lats1/2: EMT and Cellular Senescence

Lats1 and 2 phosphorylate the transcriptional cofactors Yap/Taz, and transcriptionally regulate gene expression via the Hippo pathway. Lats1/2 also regulate some transcription factors or cofactors that are involved in different signaling pathways. Specifically, Lats2 interacts with, and directly phosphorylates, a zinc-finger type-transcriptional factor called Snail1, which regulates the induction of epithelial-to-mesenchymal transitions (EMT) by repressing E-cadherin expression during embryonic development and tumor progression (Zhang et al. 2011). Lats2-mediated phosphorylation of Snail1 on T203 increases the stability and nuclear retention of Snail1. Interestingly, Lats2-mediated Snail phosphorylation is induced not only by environmental EMT stimuli such as TGFβ, but also by nocodazole-induced mitotic stress, RasV12-induced oncogenic stress, and upon activation of the Hippo pathway under high cell density conditions or upon overexpression of Mst2/WW45 (Zhang et al. 2011). Overexpression of Lats1 prevents Yap-induced EMT-like phenotypes, such as cell migration and colony formation, whereas overexpression of Lats2 has little effect on Yap-mediated EMT (Zhang et al. 2008). These results suggest the possibility that two parallel pathways for EMT regulation operate in mammalian cells; namely Lats1 represses Yap-mediated EMT (the Lats1-Yap axis), whereas Lats2 promotes Snail1-mediated EMT (the Lats2-Snail1 axis).

Lats2 also directly interacts with a ligand-dependent transcription factor called the androgen receptor (AR), which regulates the development and maintenance of the male reproductive system and is implicated in the development of prostate cancer. The Lats2-AR complex represses androgen-induced expression of the prostate-specific antigen (PSA) gene by binding to its promoter and enhancer regions (Powzaniuk et al. 2004). However, it is unclear whether Lats1/2 can phosphorylate AR in vivo.

More interestingly, Lats2 also plays an important role in Retinoblastoma protein (pRB)-induced cellular senescence. Lats2 directly phosphorylates DYRK1A and activates its kinase activity toward the LIN52 subunit of the DREAM (DP, RB [retinoblastoma], E2F, and MuvB) repressor complex, which promotes the assembly of DREAM complexes at E2F-regulated promoters and the silencing of E2F target genes such as Cdc6 and Cdc25A (Tschöp et al. 2011). Thus, it is likely that Lats2 regulates G1 arrest and apoptosis through the Lats2-p53 pathway, and also regulates cellular senescence through the Lats2-pRb pathway in response to oncogenic stress, both of which are pivotal tumor suppressor mechanisms.

On the other hand, in senescent human cells, decreased Lats1 levels result in an irreversible cytokinetic block downstream of reactive oxygen species (ROS)-protein kinase Cδ (PKCδ) signaling, a positive feedback loop in which elevated ROS levels activate PKCδ, which in turn promotes further generation of ROS (Takahashi et al. 2006). Indeed, Lats1 is directly phosphorylated at S464 by both PKCδ and NUAK1/AMPK-related protein kinase 5 (ARK5) kinases, which triggers degradation of the Lats1 protein (Takahashi et al. 2006; Humbert et al. 2010).

Taken together, these studies show that Lats1/2 play a pivotal role in tumor suppression as central regulators of a wide variety of signaling pathways via cell cycle and mitotic checkpoints, the DNA damage checkpoint, apoptosis, EMT, cellular senescence, and the Hippo pathway.

10.4 Mob1

Mob1 (Mats in the fruit fly) is an evolutionarily conserved coactivator of the Dbf2 kinase family, which includes Dbf2 and Sid2 in yeast, Warts in fruit flies, and Lats1/2 and Ndr1/2 in mammals (Hergovich 2011). Mob1 interacts with, and activates, the Dbf2 kinase family in the MEN, SIN, and Hippo pathways in yeast, fruit flies, and humans. Moreover, Mob1 is phosphorylated by an upstream Hippo kinase family, which includes Cdc15 and Sid1 in yeast, Hippo in fruit flies, and Mst1/2 in mammals. Thus, since Mob1 is also a core component of the MEN/SIN pathway, it is suggested that Mob1 is profoundly implicated in Lats-mediated mitotic regulation, as well as in the Hippo pathway (Fig. 10.1).

Like yeast Mob1, mammalian Mob1 colocalizes with Lats1/2 at the centrosome, whereas fruit fly Mats colocalizes not only with Warts kinase, but also with cyclin E at the centrosome throughout the cell cycle. Moreover, *mats* mutations cause chromosome missegregation in developing fruit fly embryos. However, loss of *mats* does not affect the colcemid-induced mitotic spindle checkpoint (Shimizu et al. 2008). Furthermore, both T12 and T35 of human Mob1 are phosphorylated by Mst1/2 kinase during nocodazole-arrested mitosis and in the presence of okadaic acid (a protein phosphatase inhibitor) and H_2O_2. These two phosphorylated sites on Mob1 are crucial for binding to Lats1 and Ndr1, and for Lats1 autophosphorylation at S909, which plays an important role in the regulation of cell proliferation. Notably, cells expressing the double non-phosphorylated mutant (T12A and T35A) show accelerated G1/S progression and mitotic exit (Praskova et al. 2008).

Most interestingly, Mob1 localizes not only at the centrosome (spindle pole) but also at the kinetochores, where Plk1 and the CPC are also colocalized (Wilmeth et al. 2010). Moreover, Plk1 is required for Mob1 to localize at the centrosome, but not at the kinetochores, whereas the CPC is required for Mob1 to localize at the kinetochores, but not at the centrosome. Notably, loss of Mob1 leads to a delay in the translocation of the CPC and mitotic kinesin-like protein 2 (Mklp2) to the central spindle/spindle mid-zone during anaphase, thereby preventing recruitment of Mklp1 to the central spindle (Wilmeth et al. 2010). These results suggest that Mob1

regulates the CPC through Lats1/2 activity at the mitotic apparatus (centrosome, kinetochore, and central spindle) during metaphase/anaphase transition. Consistent with this, Lats1/2 colocalizes with Aurora B, a catalytic component of the CPC, at the central spindle and at the chromosome arms, including the kinetochores (Yabuta et al. 2011).

10.5 Rassf1A

In the Hippo pathway, the fruit fly Ras association domain family (Rassf) and its mammalian homolog, Rassf1A, modulate the Hippo and Mst1/2 kinases, respectively. Since human *RASSF1A* is often inactivated in solid tumors, Rassf1A is thought to be a predominant candidate tumor suppressor (Donninger et al. 2007; Avruch et al. 2009). Importantly, mammalian Rassf1A regulates the cell cycle and mitosis (Fig. 10.1).

Rassf1A colocalizes with Mst2, WW45, and Lats1 at the centrosome throughout the cell cycle, and at the midbody during cytokinesis. Like Lats1/2 knockout mice, MEFs from Rassf1A knockout mice (*Rassf1A*$^{-/-}$) show a failure of cytokinesis, which can be rescued by either expression of Mst2, WW45, or Rassf1A itself (Guo et al. 2007). The centrosomal localization of Rassf1A seems to be maintained via its interaction with C19ORF5/MAP1S, which contains two conserved microtubule-associated regions (Dallol et al. 2007). Interestingly, Rassf1A associates with, and is directly phosphorylated at T202 and S203 by, Aurora A during mitosis. Although T202 and S203 are located within the microtubule-binding domain of Rassf1A, it is not clear whether these phosphorylations are involved in its interaction with microtubules (Dallol et al. 2007; Song et al. 2009b). Moreover, Rassf1A interacts with Cdc20, an activator of the anaphase promoting complex/cyclosome (APC/C), thereby inhibiting APC/C-Cdc20 function during early mitosis. Aurora A-mediated phosphorylation of Rassf1A on S203 induces its dissociation from Cdc20 and the subsequent activation of the APC/C-Cdc20 complex, thereby promoting prometaphase progression (Chow et al. 2012). Alternatively, Rassf1A interacts with DNA damage-binding protein 1 (DDB1) and is degraded by the DDB1-CUL4A E3 ligase complex during mitosis (Jiang et al. 2011).

On the other hand, Aurora B also interacts with, and phosphorylates, Rassf1A on S203; however, this occurs at different times and at different subcellular locations during mitosis. S203-phosphorylated Rassf1A colocalizes with Aurora A at the centrosome during early mitosis, whereas it colocalizes with Aurora B at the central spindle/spindle mid-zone and at the midbody during late mitosis (Song et al. 2009b). Aurora B-mediated S203 phosphorylation of Rassf1A seems to regulate cytokinesis through the interaction of Rassf1A with Syntaxin16, a member of the t-SNARE family, at the spindle mid-zone and midbody (Song et al. 2009a).

Taken together, it is likely that Rassf1A is differentially regulated by Aurora A at the centrosome to progress early mitosis, and by Aurora B at the spindle mid-zone and midbody for cytokinesis.

10.6 Other Regulators: Kibra and Ndr1/2

A WW domain-containing protein called Kibra was recently identified as a new upstream regulator within the Hippo pathway (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010). Human Kibra interacts with Lats2 to positively regulate Lats2 protein stability in the Hippo pathway (Xiao et al. 2011a). In human cancer cells such as HeLa and MCF7, Kibra is phosphorylated in a cell cycle-dependent manner, and this phosphorylation is apparently enhanced during mitosis (Xiao et al. 2011b). Notably, Kibra is directly phosphorylated at S539 by the Aurora A and Aurora B kinases and dephosphorylated by PP1. Phosphorylation at S539 is required for the interaction between Kibra and Aurora A, and for its dissociation from neurofibromatosis type 2 (NF2)/Merlin during mitosis, which is involved in nocodazole-induced mitotic arrest and mitotic exit (Xiao et al. 2011b). Taken together, these studies suggest that Kibra may play a vital role as a switch between Merlin-mediated Hippo signaling and Aurora-mediated mitotic signaling.

Ndr1 (nuclear-Dbf2-related 1) and Ndr2 belong to a subfamily that is similar to, but distinct from, the Lats1/2 subfamily within the Dbf2 superfamily. The Ndr kinase subfamily is also evolutionarily conserved from yeast to humans, e.g., Cbk1 and Orb6 in budding yeast, Sax-1 in nematodes, Trc in fruit flies, and Ndr1/2 in mammals, which all play more predominant roles in maintaining cell polarity or during morphogenesis than they do in apoptosis and cell proliferation (Hergovich and Hemmings 2009). Moreover, unlike Lats1/2, Ndr1/2 cannot phosphorylate endogenous Yap/Taz proteins in cells, although they can phosphorylate recombinant Yap protein in vitro (Hao et al. 2008). Thus, Ndr1/2 are not components of the canonical Hippo pathway. Nevertheless, it is interesting that Ndr1/2, like some regulators of the Hippo pathway, are also directly phosphorylated by Mst1/2 and function in some mitotic events, such as centrosomal and mitotic chromosomal integrity, in response to Mst1/2 kinase activity (Fig. 10.2). Specifically, Ndr1 regulates centrosome duplication through Mst1-mediated phosphorylation (Hergovich et al. 2009), whereas Ndr1 regulates mitotic chromosome alignment through Mst2-mediated phosphorylation (Chiba et al. 2009). Similar to the function of Mst1, Ndr1 associates with Aurora B and inhibits its kinase activity, which promotes the destabilization of kinetochore-microtubule attachments (Oh et al. 2010). Moreover, Ndr1/2 directly phosphorylates p21, a cyclin/Cdk inhibitor, downstream of the third Mst kinase, Mst3, which plays an important role in G1/S progression (Cornils et al. 2011a, b).

Acknowledgements We thank Dr. Moshe Oren and Dr. Yael Aylon for inviting us to write this review. We also thank Dr. Patrick Hughes and Dr. Stephen Cooke (Bioedit Ltd.) for critically reading the manuscript.

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Part V
Mouse Models

Chapter 11
Hippo and Mouse Models for Cancer

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Abstract Among the many signaling pathways related to cancer initiation and progression, the Hippo pathway has emerged recently as a mediator of tumor suppression that is evolutionarily conserved from flies to humans and plays a key role in normal organ development. Genetic engineering of the Hippo pathway in mice has provided important insights into its tumor suppression function. These mouse models have also revealed both canonical and noncanonical modes of action for pathway components in tumor suppression. In this chapter, we first discuss genetic and epigenetic changes identified for Hippo pathway components in human cancers. We then describe established mouse models of cancer related to the Hippo pathway, dividing them into those in which the canonical pathway functions through inhibition of the transcriptional co-activator YAP and those in which noncanonical functions of individual pathway components contribute to tumor suppression.

Keywords Canonical Hippo pathway • Noncanonical Hippo pathway • Liver cancer • Oval cell • Intestine cancer • Tissue regeneration • Lymphoma • Genomic instability

11.1 Introduction

Cancer develops as a result of dysregulation of multiple genes and associated signaling pathways. The accumulation of genetic and epigenetic changes that favor uncontrolled cell proliferation and spread is the driving force that advances tumor development, from initial tumor formation to escape from surrounding local tissue,

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_11, © Springer Science+Business Media New York 2013

angiogenesis, and the acquisition of resistance to detrimental elements of the tumor environment such as anticancer agents (Hanahan and Weinberg 2000, 2011). It is thus important to characterize these changes for all stages of cancer development and for all types of cancer.

The Hippo signaling pathway, first discovered a decade ago by genetic screening in *Drosophila* (Saucedo and Edgar 2007; Harvey and Tapon 2007; Zhao et al. 2010a), has recently been found to mediate tumor suppression in mammals. The molecular roles of this signaling pathway have been described in detail in other chapters of this book. According to the current simplified model, upstream components of the Hippo pathway include Kibra, Merlin, and Expanded. The core complex of the pathway consists of the protein kinase Hippo (MST1 and MST2 in mammals), Salvador (SAV1 or WW45 in mammals), Mats (MOB1 in mammals), and the protein kinase Warts (LATS1 and LATS2 in mammals). On activation by unknown signals, Hippo phosphorylates and thereby activates Warts with the help of Salvador. Mats binds to Warts and enhances its kinase activity. Activated Warts, in turn, phosphorylates and inactivates the transcriptional co-activator Yorkie (YAP and TAZ in mammals). The mammalian counterparts of the Hippo signaling pathway in *Drosophila* are both molecularly and functionally well conserved.

11.2 Dysregulation of Hippo Pathway Components in Human Cancer

Inactivation or reduced expression of upstream regulators of YAP has been identified in human cancers, as has activation of YAP. Hippo pathway components whose expression has been found to be dysregulated in human cancers and the mechanistic basis for such altered expression are summarized in Table 11.1.

The gene for NF2 (Merlin) is the most frequently mutated of the genes for Hippo pathway components. It is thus mutated in individuals with familial neurofibromatosis type 2 (NF2) (Trofatter et al. 1993), which is characterized by the development of multiple tumors of the nervous system such as schwannoma, meningioma, and ependymoma. Mutations in NF2 often result in the generation of truncated proteins, although several missense mutations have been associated with less aggressive forms of the disease (Ahronowitz et al. 2007; Baser 2006). NF2 mutations have also been found in sporadic neuronal tumors. Importantly, both familial and sporadic cancers manifest loss of heterozygosity (LOH) for NF2, which is often seen with tumor suppressor genes, therefore suggesting that NF2 is indeed an authentic tumor suppressor. Epigenetic changes for NF2 have not been detected in human tumors to date. Direct mutation therefore appears to be the major mechanism for disruption of NF2 function in cancer. In addition to neuronal tumors, a high frequency of NF2 mutations has been detected in mesothelioma, a metastatic type of cancer originating from epithelial cells that line the abdominal cavity (Bianchi et al. 1995).

Table 11.1 Alterations of Hippo pathway genes in human cancer

Gene	Dysregulated cancer	Alteration mechanism
NF2	Familial neurofibromatosis type 2 Sporadic neurofibromatosis type 2 Mesothelioma	Nonsense/frameshift/point mutations. LOH found
MST1/2	Soft tissue sarcoma	Promoter methylation
LATS1/2	Non-small cell lung cancer	13q12 loss (67 %). Rare inactivating mutations
	T-ALL	13q12 loss (3–6 %), promoter methylation
	Astrocytoma	Promoter methylation
	Breast cancer	Promoter methylation
	Mesothelioma	13q12 loss, inactivating mutations
YAP	Glioblastoma, Oral SCC, pancreas, cervix, lung, liver, mesothelioma	11q22 amplification (5–10 %) Immunohistochemical analysis indicate accumulation of YAP/TAZ in higher portion

Mutational inactivation of MST1 or MST2 has not been identified to date in human cancer, possibly because their functional redundancy would necessitate disruption of both genes. Indeed, Mst1-null and Mst2-null mice are viable, appear to develop normally, and rarely manifest spontaneous tumors, indicative of the functional redundancy of the two proteins (Oh et al. 2009; Zhou et al. 2009). It is therefore unlikely that mutational inactivation of both MST1 and MST2 would serve as the initiating lesion for tumorigenesis. Nevertheless, reduced expression of MST1 and MST2 may promote tumor progression, as suggested by the frequent methylation of both gene promoters in soft tissue sarcoma (Seidel et al. 2007).

The promoters of *LATS1* and *LATS2* also undergo extensive methylation in various types of cancer. In the case of T-cell acute lymphoblastic leukemia (T-ALL), breast cancer, and astrocytoma, more than 50 % of tumors manifest *LATS1* or *LATS2* promoter methylation (Jimenez-Velasco et al. 2005; Morinaga et al. 2000; Jiang et al. 2006), with the extent of methylation correlating negatively with LATS1/2 expression and prognosis. Of note, LOH at chromosome 13q12, a locus that includes *LATS2*, has also been detected in T-ALL (3–6 %), lung cancer (67 %), mesothelioma, and cancers of the liver and ovary (Jimenez-Velasco et al. 2005; Yokota et al. 1987; De Rienzo et al. 2000). Rare inactivating mutations of LATS2 have also been identified in lung cancer and mesothelioma (Strazisar et al. 2009; Murakami et al. 2011). The fact that loss of LATS1 or LATS2 expression (or both) is frequently observed in human tumors suggests that the two proteins may perform distinct tumor suppressor functions in different contexts. Indeed, in contrast to Mst1 and Mst2 single-knockout mice, Lats1 and Lats2 single-knockout mice have distinct phenotypes characterized by the spontaneous formation of soft tissue sarcomas and embryonic lethality, respectively (St John et al. 1999; McPherson et al. 2004).

YAP and TAZ are the main downstream targets of the Hippo pathway in mammals and function as oncogenic proteins. Both YAP and TAZ are inactivated as a result of LATS-mediated phosphorylation, leading to their cytoplasmic sequestration or degradation (Zhao et al. 2010b; Dong et al. 2007; Lee et al. 2008). However, no activating missense mutations of YAP or TAZ have been identified in human cancer to date. Instead, the activity of YAP is increased as a result of its increased expression and nuclear localization in certain cancers. For example, amplification of chromosome 11q22, which harbors the genes for YAP and the anti-apoptotic protein cIAP1, has been detected in 5–10 % of glioblastomas, oral squamous cell carcinomas, mesotheliomas, and cancers of the cervix, pancreas, breast, lung, and liver (Baldwin et al. 2005; Li et al. 2012; Hermsen et al. 2005; Imoto et al. 2001, 2002; Snijders et al. 2005; Weber et al. 1996). Moreover, immunohistochemical studies indicate that overexpression of YAP or TAZ occurs in a much higher proportion of tumors (Zhao et al. 2007). Amplification of the 11q22 locus may thus account for YAP activation in only a subset of tumors, with other mechanisms of YAP accumulation, such as those mediated at the transcriptional or translational level, waiting to be identified.

11.3 Tumor Suppression by the Canonical Hippo Pathway in Mouse Models

Among the first mouse models to suggest the importance of the Hippo pathway in cancer were YAP transgenic mice generated by two independent groups (Dong et al. 2007; Camargo et al. 2007). These transgenic mice provided insight into two in vivo functions of the Hippo pathway in mammals: (1) Control of organ size. The two groups thus both found a marked increase in organ size in the transgenic animals. The size of the liver returned to normal when expression of the YAP transgene was eliminated. (2) Control of stem or progenitor cell proliferation and differentiation. The size of stem/progenitor cell compartments in various organs including the intestine and skin was thus increased in YAP transgenic mice, suggesting that the Hippo pathway limits stem or progenitor cell proliferation and promotes cell differentiation (Camargo et al. 2007; Schlegelmilch et al. 2011).

Subsequent studies focused on the precise roles of individual Hippo pathway components with regard to these two functions. Knockout mice with mutations in the genes for each component have thus been generated (Oh et al. 2009; McPherson et al. 2004; Lee et al. 2008; McClatchey et al. 1997). However, embryonic mortality of mice lacking Nf2, Sav1, Lats2, or both Mst1 and Mst2 has hampered investigations into the roles of the Hippo pathway in tumorigenesis. Tissue-specific knockout mice have been generated to overcome such mortality. Studies that have linked loss of Hippo signaling to liver, intestinal, and other types of cancer will be discussed.

11.3.1 The Hippo Pathway in Liver Cancer

The liver serves as an ideal system for studies of the control of organ size. Each individual maintains a constant size of the liver; even after severe insults such as partial hepatectomy of up to two-thirds of the tissue, the liver undergoes rapid regeneration to regain its original size. This unique feature of the liver has prompted many studies into the potential role of the Hippo pathway in this organ.

The mammalian liver is composed of two major differentiated cell types: hepatocytes and cholangiocytes (Roskams 2006). In a typical epithelial tissue, increased proliferation of stem or progenitor cells (but not of differentiated cells) is largely responsible for tissue regeneration associated with the replacement of old or damaged cells. In contrast, even though hepatocytes usually remain quiescent and rarely divide under normal conditions, they are able to undergo massive proliferation to replace damaged cells after extensive tissue injury. Only if replication of hepatocytes is blocked by hepatotoxins or if the extent of tissue damage exceeds the regenerative capacity of these cells do hepatic stem/progenitor cells, the so-called oval cells, become activated and divide to give rise to both hepatocytes and cholangiocytes. Oval cells normally reside in peripheral regions of the biliary tree known as the canals of Hering, and they rarely proliferate in the absence of severe liver damage. Importantly, many risk factors for human liver cancer, including infection with hepatitis B or C viruses as well as alcoholic or nonalcoholic steatohepatitis, can lead to oval cell activation, suggesting that oval cells are a candidate cell-of-origin for some liver cancers (Roskams 2006; Farazi and DePinho 2006).

The two most common types of liver cancer are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). A mixed type (having both HCC and CC characteristics) and an intermediate type (having ill-defined characteristics) of liver cancer also exist and are thought to originate from liver stem/progenitor cells. Some individuals with HCC also have transformed hepatocytes that express classic oval cell markers such as CK19, with such expression correlating with poor prognosis, suggesting that these cancers could originate from either oval cells or dedifferentiated transformed hepatocytes.

The Hippo pathway was first implicated in liver cancer by an unbiased genome-wide screen for new cancer genes (Zender et al. 2006). In this study, combinations of human oncogenes were introduced into hepatoblasts isolated from mouse embryos and the cells were then transplanted into the liver of normal mice. The locus including the *Yap* and *cIAP1* genes was found to be recurrently amplified in tumors induced by the *c-MYC* oncogene. This locus is syntenic to human chromosome 11q22, which is also amplified in a subset of human cancers (as described above). Transgenic mice that overexpress YAP specifically in the liver were subsequently found to develop hepatomegaly followed by HCC (Dong et al. 2007; Camargo et al. 2007). These studies thus provided direct evidence for an oncogenic function of YAP in the liver. In addition, recurrent amplification of the genomic locus containing *Yap* was identified in breast tumors of $Brca1^{+/-};p53^{+/-}$ mice, providing further support for such a function of YAP in another system (Overholtzer et al. 2006).

Can ablation of upstream negative regulators of YAP also induce liver cancer in the absence of exogenous YAP? So far, the genes for *Nf2*, *Sav1*, and both *Mst1* and *Mst2* have been deleted in the liver to address this question (Zhou et al. 2009; Zhang et al. 2010; Benhamouche et al. 2010; Lee et al. 2010; Song et al. 2010; Lu et al. 2010). All three mouse strains exhibited hepatomegaly and ultimately developed liver cancer with a time course similar to or slower than that for liver-specific YAP transgenic mice. The extent of Yap phosphorylation in the liver was markedly reduced in these models, and, as a result, Yap accumulated to high levels in the nucleus. Importantly, deletion of one allele of *Yap* in mice lacking *Nf2* in the liver abolished hepatomegaly and tumor formation (Zhang et al. 2010). Control mice with only one *Yap* allele showed no defect in liver development or homeostasis. Similarly, growth of HCC cell lines derived from mice lacking both Mst1 and Mst2 in the liver was also inhibited by knockdown of *Yap* (Zhou et al. 2009). These genetic studies thus demonstrated that inactivation of upstream components of the Hippo pathway can initiate liver tumorigenesis via YAP activation.

Ablation of *Nf2* or *Sav1* specifically in the liver resulted in the selective overproliferation of immature cells that were likely oval cells, without any marked effect on differentiated hepatocytes (Benhamouche et al. 2010; Lee et al. 2010). Oval cell hyperplasia is also induced by hepatocyte damage, but the knockout mice exhibited no apparent defects in hepatocytes, suggesting that the oval cell proliferation in these animals was not due to liver damage. The liver tumors that developed in these *Nf2*- or *Sav1*-deficient mice with age were the mixed type, with characteristics of both HCC and CC. In recent, however, many more analysis of the liver tumors derived from *Sav1*-deficient mice had let us notice that Sav1-null mice also frequently developed only HCC with some progenitor expansion (T.-S.K. and D.-S.L., personal observation). Nonetheless, these findings, together with the preceding oval cell hyperplasia, suggested that the tumors could be derived from oval cells. NF2 and SAV1 in the Hippo pathway thus appear to inhibit liver tumorigenesis by restricting liver stem/progenitor cell proliferation.

The liver-specific *Nf2*-null and *Sav1*-null mice have also provided evidence that liver damage is linked to tumorigenesis through oval cell activation. Oval cell activation in these mice was thus enhanced further by hepatocyte damage induced either by a diet containing the hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or by partial hepatectomy. Liver-specific *Sav1* knockout mice thus responded to short-term consumption of a DDC diet with excessive oval cell expansion. More dramatic results were obtained with *Nf2* knockout mice. When deletion of *Nf2* in the liver was induced postnatally by injection of an adenoviral vector for Cre recombinase or by interferon-driven Cre expression, only mild periportal hyperplasia ensued and macroscopic tumors did not develop. These findings thus contrasted with the pronounced oval cell hyperplasia and subsequent tumor development observed in the mice in which *Nf2* was deleted during early liver development in embryos as a result of Cre expression controlled by the albumin gene promoter. However, surgical removal of two-thirds of the liver to induce liver regeneration in the two former mouse models resulted in marked overproliferation of oval cells and development of

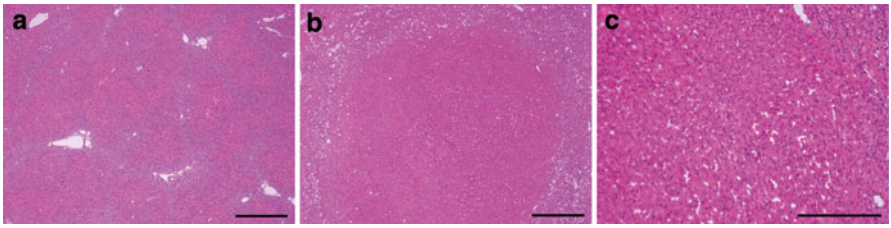

Fig. 11.1 Liver cancer in albumin Cre; $Mst1^{flox/flox}$; $Mst2^{-/-}$ mice. H&E-stained liver sections from the mice deficient for liver Mst1 and Mst2. Liver-specific gene deletion was achieved by mating with albumin-Cre transgenic mice. (**a**) A picture of liver from 9-week-old knockout mice showing abnormal architecture with increased progenitor-like cells around portal triads. (**b**) A representative picture of an HCC node developed in 6-month-old knockout mice. The progenitor-like cells were set aside the tumor node. (**c**) High magnification view of (**b**). Scale bars indicate 500 μm for (**a**) and (**b**), and 200 μm for (**c**)

mixed HCC-CC tumors, as observed in the albumin-Cre model without hepatectomy. The acceleration of tumor development by regenerative stimuli thus again highlighted the importance of regulation of liver stem/progenitor cells by the Hippo pathway. Moreover, these findings are also clinically relevant given that, as mentioned above, risk factors for liver cancer in humans (hepatitis B or C virus infection, steatohepatitis) are linked to chronic liver damage or inflammation and ultimately to oval cell activation (Roskams 2006; Farazi and DePinho 2006). Although oval cell reactions are observed in some human liver tumors, few animal models have been available to examine their impact on actual tumorigenesis. Liver-specific *Nf2* or *Sav1* knockout mice thus represent important tools to study the role of oval cells in liver cancer.

Deletion of *Mst1* and *Mst2* in the liver also led to tumor formation (Zhou et al. 2009; Song et al. 2010; Lu et al. 2010), which was more rapid than that in liver-specific *Nf2* or *Sav1* knockout mice (Fig. 11.1). Strikingly, unlike these latter mice, most liver tumors formed in mice with only one copy of *Mst1* or *Mst2* were classified as HCC, with only a minor fraction being classified as CC or mixed HCC-CC. Complete inactivation of *Mst1* and *Mst2* by expression of Cre recombinase under the control of the albumin gene promoter resulted in overproliferation of both hepatocytes and oval cells followed by the development of large liver tumors. Again, most of these tumors exhibited histological characteristics of HCC, with a smaller proportion of mixed HCC-CC tumors also being detected. These *Mst1/2* knockout mice also appeared to have liver damage, as evidenced by high levels of alanine and aspartate aminotransferases in their serum and inflammatory gene expression profile in their liver (Song et al. 2010; Lu et al. 2010). This liver damage might explain why tumor initiation in these *Mst1/2* knockout mice was more rapid than that in *Sav1* knockout mice. These observations suggested that, unlike NF2 and SAV1, MST1 and MST2 function as potent tumor suppressors in the hepatocyte compartment. Although it remains possible that MST1 and MST2 regulate oval cell homeostasis, liver damage or inflammation in animals with liver-specific ablation of these proteins may also

contribute to and accelerate liver tumorigenesis. Notably, *Mst1*- and *Mst2*-deficient hepatocytes showed a markedly reduced level of Yap phosphorylation on serine-127, the residue targeted by Lats1/2 kinases, likely resulting in up-regulation of Yap's oncogenic activity. Whereas Yap's transgenic mice develop HCC, whether these animals also show expansion of the oval cell compartment has not been described (Dong et al. 2007; Camargo et al. 2007). In the future, it will be important to determine the relative contributions of hepatocytes and oval cells to liver tumorigenesis in liver-specific *Mst1* and *Mst2* double-knockout mice.

11.3.2 The Hippo Pathway in Intestinal Cancer

The intestine harbors relatively well-characterized stem cells, which are located at the base of intestinal crypts and turn over rapidly to compensate for the abrasion-induced loss of epithelial cells in the lumen and thereby maintain homeostasis (van der Flier and Clevers 2009). YAP transgenic mice rapidly develop severe intestinal dysplasia with the near complete loss of differentiated cells (Camargo et al. 2007), whereas systematic Sav1 knockout embryos exhibit expansion of progenitors and defects in cell differentiation in the intestine (Lee et al. 2008). These observations implicate the Hippo pathway in intestinal stem cell regulation and intestinal cancer.

Conditional knockout mice lacking Mst1 and Mst2 in the intestine manifest a phenotype essentially corresponding to that of the liver-specific double-knockout mice (Zhou et al. 2011). Similar to the effects of YAP overexpression, deletion of *Mst1* and *Mst2* in the intestinal epithelium thus induced enlargement of crypts in the small intestine and dysplasia of the colon (Fig. 11.2). At the molecular level, both Wnt and Notch signaling pathways (which drive proliferation of stem and progenitor cells, respectively) were activated in the intestine of these mice. The expansion of stem and progenitor cell compartments was accompanied by a marked reduction in the number of differentiated cells in the intestine of the mutant animals. The extent of Yap phosphorylation was also reduced in association with the nuclear accumulation of Yap in intestinal cells of the double-mutant mice. Furthermore, deletion of one *Yap* allele in these animals rescued the cell proliferation–differentiation phenotype, confirming the role for the canonical Hippo pathway in stem-progenitor cell regulation.

In contrast to *Mst1/2* deletion, deletion of *Sav1* in the intestine had no impact on intestinal homeostasis, with the exception that aged mice developed mild hyperplasia in the colon (Cai et al. 2010). However, treatment of these *Sav1*-deficient mice with dextran sulfate sodium (DSS), which damages the colonic epithelium, resulted in an exaggerated regenerative response and subsequent polyp formation. Again, deletion of one *Yap* allele abolished this hyper-regenerative response. DSS treatment in the wild-type mice also resulted in the rapid accumulation of Yap in the

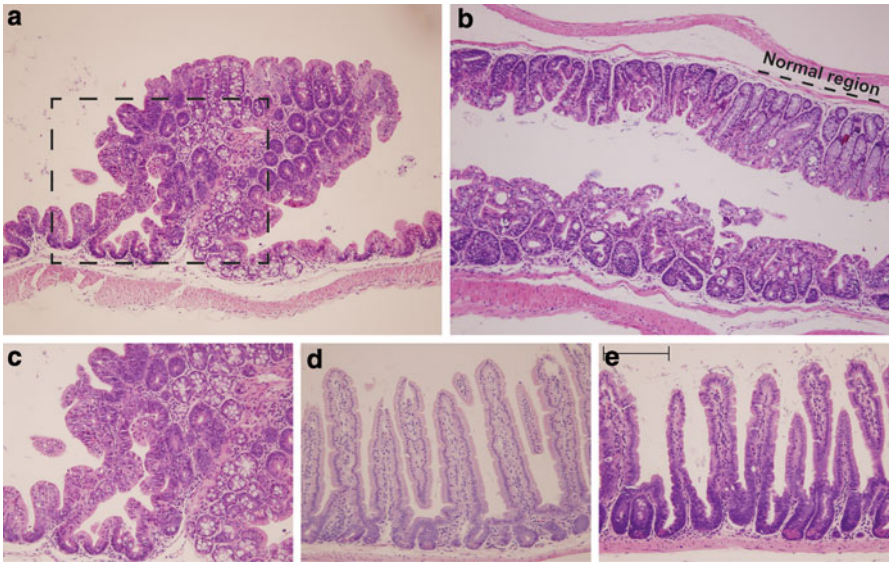

Fig. 11.2 Small and large intestine in Villin Cre; mice; $Mst1^{flox/flox}$; $Mst2^{-/-}$ mice. H&E-stained colon and small intestine sections from the mice deficient for intestine Mst1 and Mst2. Intestine-specific gene deletion was achieved by mating with Villin-Cre transgenic mice. (**a**–**c**) Adenomas developed in 2-week-old Mst1/2 intestine-specific knockout mice. (**a**) Polyp type adenoma formed in colon of Villin Cre; Mice; $Mst1^{flox/flox}$; $Mst2^{-/-}$ mouse. The enlarged image of inset is shown in (**c**), an aggressive part with high proliferation at the base and loss of differentiated cells. Right part of the polyp is relatively less transformed, which maintains differentiated villi structure. (**b**) Flat type adenoma formed. *Dashed line* indicates regions maintaining normal architecture of the colon. Left to the indicate region, the normal columnar architecture of colon is lost, accompanied by proliferation at the base and loss of differentiated goblet cells. (**d**, **e**) Small intestine of control (**d**) and Villin Cre; Mice; $Mst1^{flox/flox}$; $Mst2^{-/-}$ mouse (**e**) at 2-weeks of age. The small intestine maintains normal architecture at this age. However, the size of the crypt compartments, which contains the stem/progenitor cells, is extremely enlarged in Villin Cre; Mice; $Mst1^{flox/flox}$; $Mst2^{-/-}$ mouse

intestinal epithelium followed by normalization of Yap expression as regeneration was completed. This finding suggests that YAP contributes to the regenerative response to tissue damage. Furthermore, the absence of SAV1 or of MST1/2 likely results in the constitutive activation of YAP, which leads to continuous tissue regeneration and the consequent development of hyperplasia and cancer. The central role of YAP in intestinal regeneration was confirmed by the production of mice lacking Yap in the intestine, which failed to replace damaged tissue and died soon after DSS treatment (Cai et al. 2010). Of note, these animals showed no developmental defects in the intestine, indicating that YAP is dispensable for intestinal development but indispensable for regeneration of the intestine after injury.

11.4 Tumor Suppression by Noncanonical Functions of Hippo Pathway Components

11.4.1 Role of the MST1-SAV1-NDR1 Signaling Axis in Maintenance of Genomic Stability

In addition to their role in regulation of cell proliferation and differentiation through YAP, the core Hippo pathway components are implicated in regulation of the cell cycle. The LATS-MOB1 complex thus has an evolutionarily conserved role in mitotic exit and centrosome maintenance (Bothos et al. 2005; Brace et al. 2011). Recent studies also indicate that the MST1-SAV1-NDR1 axis performs multiple cell cycle functions. The protein kinase NDR1 is a paralog of LATS1/2, and MST1-NDR1 signaling promotes stable kinetochore-microtubule attachment by restraining Aurora B activity and centrosome duplication, whereas the MST1-SAV1 complex regulates centrosome disjunction via Nek2A (Oh et al. 2010; Hergovich et al. 2009; Mardin et al. 2010). Defects in any of these cell cycle events ultimately lead to incorrect chromosome segregation to daughter cells and aneuploidy. Although still controversial, increasing evidence suggests that aneuploidy and chromosomal instability contribute to tumor initiation and progression (Kops et al. 2005). Analysis of the hematopoietic system of Mst1-null mice has provided support for MST1 function in maintenance of chromosome integrity. These mice were thus found to be highly susceptible to the development of *N*-ethyl-*N*-nitrosourea (ENU)-induced T-ALL (Kim et al. 2012). Interestingly, Mst1-deficient lymphocytes from these mice showed a normal proliferation rate and susceptibility to pro-apoptotic stimuli. Moreover, Mst1 deficiency did not affect mouse lymphocyte developmental programs, even though naïve mouse Mst1-null T cells or human MST1-null lymphocytes undergo spontaneous apoptosis (Choi et al. 2009; Nehme et al. 2012). Rather, mouse Mst1-deficient lymphocytes manifested an increased frequency of abnormal mitosis and genomic instability, and ENU-induced lymphomas in Mst1-null mice therefore also exhibited a high incidence of genomic instability. Most Mst1-null lymphocytes that undergo abnormal mitosis would be expected to be eliminated as a result of activation of the p53-dependent cell death pathway. Consistent with this notion, Mst1 deficiency and *p53* deletion induced a markedly synergistic increase in the incidence of T cell lymphoma.

Ndr1 knockout mice, similar to Mst1 knockout mice, show an increased susceptibility to ENU-induced lymphoma (Cornils et al. 2010). Although ablation of *Ndr1* conferred a subtle protection from apoptosis, defective mitosis in lymphocytes is likely to contribute to the increased tumor incidence in this model. In this regard, whereas overexpression of either wild-type or a constitutively active form of YAP in epithelial tissues resulted in tumor development, that in the hematopoietic system had no apparent effect on the size, proliferation, or differentiation of the stem cell population (Jansson and Larsson 2012). The MST1-NDR1 axis thus appears to execute a tumor suppressor function independent of YAP in the hematopoietic system.

Like their paralog NDR1, LATS1 and LATS2 play an essential role during mitosis. Lats2-deficient mouse embryonic fibroblasts are characterized by cytokinesis failure, increased ploidy, and an accelerated exit from mitosis (McPherson et al. 2004). The rapid proliferation of these cells likely contributes to the generation of progeny with abnormal ploidy. The failure of cytokinesis and increased proliferation rate are even more pronounced in Lats1/2 double-knockout cells (M.-C.K. and D.-S.L, unpublished data). It remains to be determined whether Lats1 and Lats2 single-knockout or Lats1/2 double-knockout mice manifest an increased frequency of aneuploidy, and if so whether such aneuploidy might contribute to tumorigenesis. Furthermore, determination of the relative contributions of two different outcomes of Lats1/2 deletion—mitosis failure and YAP activation—to tumorigenesis in vivo will be important for a full understanding of the tumor suppressor function of LATS.

11.4.2 Other Knockout Mouse Models of Cancer

Even though NF2 acts as a classic tumor suppressor gene, such a role has not yet been linked to the Hippo pathway in certain tissues. For example, *Nf2* heterozygous mice show an increased susceptibility to asbestos-induced mesothelioma (Fleury-Feith et al. 2003). It is possible that dysregulation of the Hippo pathway is responsible for this sensitivity, given that mutations in various Hippo pathway components are associated with mesothelioma. In human mesothelioma, NF2 mutation, 13q12 deletion, or one of several inactivating mutations of LATS2, or 11q22 amplification and associated YAP activation are found (Murakami et al. 2011; Bianchi et al. 1995; Thurneysen et al. 2009; Mizuno et al. 2012). In vitro studies indicate that reconstitution of the canonical Hippo pathway suppresses the tumorigenic potential of mesothelioma cell lines harboring mutations in Hippo pathway components (Mizuno et al. 2012). The generation of mouse models of mesothelioma with mutations of Hippo pathway components should provide insight into the tumor suppressor function of this pathway in mesothelioma development.

Mice lacking Nf2 in the intestine and kidney have been generated by expression of Cre recombinase under the control of the *Villin* gene promoter. However, these animals were found to develop only renal cell carcinoma (Morris and McClatchey 2009). It will be of interest to examine further whether *Nf2* deletion in the intestine has any impact on intestinal homeostasis and tissue regeneration after injury.

Additional mouse models with genetic modification of Hippo pathway components and their phenotypes are listed in Table 11.2. Many such models develop various tumors, the mechanisms of which require further clarification. For example, Lats1 knockout mice develop soft tissue sarcomas with a high penetrance as well as ovarian stromal cell tumors (St John et al. 1999). RASSF family proteins, putative activators or inhibitors of MST, also serve as tumor suppressors. *Rassf1A*-null mice develop various tumors including lung adenoma, lymphoma, and breast adenocarcinoma at advanced ages (Tommasi et al. 2005; van der Weyden et al. 2005), whereas *Rassf5*-null mice did not show any substantial increase in the

Table 11.2 Reported models and phenotypes of Hippo pathway mutant mice strains

Gene	Model	Phenotype	References
NF2	Nf2 heterozygote	(1) Osteosarcoma, fibrosarcoma, low frequency HCC with LOH. Although human NF2 patients develop benign tumors in restricted tissues, NF2 mice develop much broader spectrums of aggressive cancers	McClatchey et al. (1998)
		(2) Sensitive to asbestos-induced mesothelioma development	Fleury-Feith et al. (2003)
	P0-Cre; Nf2 fl/fl	Develops schwannoma, mimics human NF2	Giovannini et al. (2000)
	P0::Nf2 ΔE2	Expression of NF2 ΔExon2 gene under P0 promoter. Develops schwannoma. Implicates dominant-negative function of this patient form of NF2	Giovannini et al. (1999)
	Nf2 fl/fl Adeno-Cre injection (leptomeningeal cell)	Develops meningioma, mimics human NF2. p53 status does not affect NF2 deletion-mediated meningioma development	Kalamarides et al. (2002)
	Nf2 fl/fl Adeno-Cre injection (Tail Vein)	Liver hyperplasia. Progresses to liver cancer by partial hepatectomy	Benhamouche et al. (2010)
	Mx1-Cre; Nf2 fl/fl	(1) Liver hyperplasia. Progresses to liver cancer by partial hepatectomy	Benhamouche et al. (2010)
		(2) Loss of HSC due to increased bone marrow angiogenesis and HSC leakage	Larsson et al. (2008)
	Albumin-Cre; Nf2 fl/fl	Mixed type HCC/CC liver cancer. Intermediate type liver cancer	Zhang et al. (2010) and Benhamouche et al. (2010)
	Villin-Cre; Nf2 fl/fl	Renal adenoma within 3 months. Progresses to renal cell carcinoma within 10 months	Morris and McClatchey (2009)
	Nf2 KO	Embryonic lethality due to gastrulation defect	McClatchey et al. (1997)

MST1/2	Mst1/2 double KO	Embryonic lethal between E9.5–11.5	Oh et al. (2009) and Zhou et al. (2009)
	Albumin-Cre; Mst1/2 fl/fl	Hepatocellular carcinoma. Minor frequencies of CC or HCC/CC	Zhou et al. (2009) and Lu et al. (2010)
	Mst1/2 fl/fl Adeno-Cre injection (Tail Vein)	Hepatocellular carcinoma. Minor frequencies of CC or HCC/CC	Zhou et al. (2009)
	Tamoxifen-Cre; Mst1/2 fl/fl	Hepatocellular carcinoma. Minor frequencies of CC or HCC/CC	Song et al. (2010)
	Mst1 KO	(1) Sensitive to ENU-induced lymphomagenesis. Synergizes with p53 loss to induce lymphomagenesis	Kim et al. (2012)
		(2) T cell lymphopenia due to defective T cell migration or defective ROS scavenging	Choi et al. (2009) and Katagiri et al. (2009)
	α-MHC; Mst1 KD	Heart-specific expression of Mst1 dominant negative. Basal heart and cardiomyocyte size was not affected. In model of myocardiac infarction, transgenic mice showed improved cardiac function without effect on cardiac hypertrophy	Odashima et al. (2007)
SAV1	Sav1 KO	Embryonic lethal at E17.5–18.5 due to placental defect. Hyperplasia in multiple epithelial tissues	Lee et al. (2008)
	Albulin-Cre; Sav1 fl/fl	Mixed type HCC/CC or HCC liver cancer	Lee et al. (2010) and Lu et al. (2010)
	Villin-Cre; Sav1 fl/fl	Colon hyperplasia in aged mice. Tumor promoted by DSS-induced tissue damage	Cai et al. (2010)
	Nkx2.5 Cre; Sav1 fl/fl	Hyperplastic cardiomyocyte. Die shortly after birth. YAP cooperates with β-catenin to drive cardiomyocyte proliferation	Heallen et al. (2011)

(continued)

Table 11.2 (continued)

Gene	Model	Phenotype	References
LATS1/2	Lats1 KO	Partial embryonic lethality. Surviving mice develop pituitary hyperplasia, fibrosarcoma, ovarian stromal cell cancer. Sensitive to DMBA/UVB-induced skin cancer	St John et al. (1999)
	Lats2 KO	Embryonic lethality at E10.5–12.5. Knockout MEF show loss of contact inhibition, defective mitosis	McPherson et al. (2004) and Yabuta et al. (2007)
	α-MHC; Lats2 WT and KD	Heart-specific expression of Lats2 and its dominant negative. Unlike Mst1, Lats2 seems to regulate organ size in heart. Lats2 WT Tg had decreased heart size, whereas Lats2 KD Tg had increased heart size. In addition, phenotype of Mst1 KD Tg was mediated by Lats2 as Lats2 KD transgene abolished the myocardial infarction phenotype	Matsui et al. (2008)
NDR1	Ndr1 KO	Spontaneous T-cell lymphoma in aged mice. Sensitive to ENU-induced lymphomagenesis	Cornils et al. (2010)
RASSF	Rassf1a KO	Spontaneous tumors in aged mice. Sensitive to carcinogen-induced tumorigenesis. Tumors in heterozygotes undergo LOH	Tommasi et al. (2005) and van der Weyden et al. (2005)
	Rassf2 KO	Perinatal lethality with defective bone development	Song et al. (2012)
	Rassf5 KO	(1) Defective lymphocyte and dendritic cell adhesion and migration	Katagiri et al. (2004)
		(2) Autoimmune disease and B cell lymphoma in aged mice. Nore1 (spliced form of Rassf5) was suggested to promote cytoplasmic localization of p27. Another group reported very low rate of spontaneous tumors in these mice	Park et al. (2010) and Katagiri et al. (2011)

YAP/TAZ	Yap S127A ROSA transgenic	Tumor development in multiple epithelial tissues (however, hematopoietic tissues had no phenotype)	Camargo et al. (2007)
	Yap KO	Embryonic lethality at E8.5 due to yolk sac defect	Morin-Kensicki et al. (2006)
	Taz KO	Develop renal cyst and pulmonary emphysema	Hossain et al. (2007) and Makita et al. (2008)
	Yap/Taz double KO	Embryonic lethality before morula stage (16–32 cells)	Nishioka et al. (2009)
	K14 Cre; Yap fl/fl	Defective development of skin barrier and neonatal lethality. Skin stem cell fail to proliferate	Schlegelmilch et al. (2011)
	Albumin Cre; Yap fl/fl	Liver development normally proceeds. After development, hepatocytes and cholangiocytes undergo spontaneous cell death	Zhang et al. (2010)
	Villin Cre; Yap fl/fl	Intestine development normally proceeds. Fails to regenerate colonic epithelium when challenged to DSS insult	Cai et al. (2010)
	Nkx2.5 Cre; Yap fl/fl	Developing cardiomyocytes fail to proliferate and die during development. Impaired IGF activity and β-catenin activity have been reported	Xin et al. (2011)
	Yap S89A knock-in	Defective in binding to TEAD transcription factors. Phenocopies YAP deletion in skin, which confirms essentiality of TEADs in YAP function in mammals	Schlegelmilch et al. (2011)

frequency of spontaneous tumor development (Park et al. 2010). The mechanisms underlying tumorigenesis in these RASSF knockout mice, including whether it depends on YAP or TAZ, await further investigation.

11.5 Future Directions

The establishment of various mouse models with genetic modifications of the Hippo pathway has revealed that the pathway exerts tumor suppressor activity through inhibition of YAP as well as that pathway components exert such activity independently of YAP, as in the maintenance of genomic integrity by the MST1-SAV1-NDR1 axis. We have focused mostly on models of liver/intestine cancer and lymphoma, respectively, in our discussion of these canonical and noncanonical roles of Hippo pathway components.

The first decade of research into the Hippo pathway has yielded many mechanistic and genetic insights. However, analysis of the role of Hippo pathway components in many genetic models of cancer, especially liver cancer, has led us to as many questions as answers. Characterization of the tumor suppressor role of Hippo pathway components in vitro has complemented the work with mouse models of cancer in vivo. We envision two key directions for future research into the role of the Hippo pathway in cancer development: (1) refinement of mouse models of cancer, and (2) discovery of novel mechanisms of tumor suppression by Hippo pathway components in vivo.

11.5.1 Refinement of Mouse Models of Cancer

Although mice deficient in individual Hippo pathway components, together with YAP transgenic mice, have been invaluable for modeling Hippo pathway dysregulation in human cancer, these mice have limitations that necessitate further refinement of the models to render them more clinically relevant. For example, in human cancer, the *YAP* locus is amplified as part of the 11q22 amplicon. This amplicon contains another putative oncogene, that for *cIAP1*, which has been suggested to have a synergistic effect with YAP in tumorigenesis. The accuracy of the YAP transgenic model would thus be increased by adjustment of the expression of other genes located in the 11q22 amplicon in addition to YAP to levels similar to those observed in human tumors with this amplicon. Activation of YAP or ablation of upstream regulators in a target tissue in a chimeric manner would also provide a more accurate model of human cancer, given that most such changes occur postnatally and in only a few cells within a tissue. Such models would also allow analysis of communication between mutant cells and surrounding normal cells. In addition, it will be important to examine whether *Lats2* deletion in mice can initiate tumorigenesis in organs in which 13q22 LOH is found in humans.

In addition to modeling the genetic alterations associated with human cancer, it should prove valuable to examine the role of the Hippo pathway in clinically relevant cancer-predisposing conditions. The finding that dysregulation of the Hippo pathway induces oval cell activation provides an opportunity to examine this issue in the liver. Given that most of the agents known to cause liver cancer induce oval cell activation and that the extent of this response is predictive of disease outcome, it will be of interest to establish mouse models that mimic tumor-promoting situations and then to test the role of the Hippo pathway.

11.5.2 Discovery of Novel Mechanisms of Tumor Suppression In Vivo

Liver cancer models with genetic alterations of the Hippo pathway have shown mechanistically and histologically distinct phenotypes. Deletion of *Nf2* generated mixed-type tumors with both HCC and CC characteristics. Also, deletion of *Sav1* generated either mixed-type HCC-CC or HCC with less expansion of progenitor cells, whereas deletion of *Mst1* and *Mst2* generated mainly HCC with more expansion of progenitor cells. These observations suggest that these genes differ in their actions in different cell lineages. A more thorough examination of the various knockout mice, together with specific ablation of Hippo pathway components in specific cell lineages (such as differentiated hepatocytes or oval cells), may provide an explanation for this difference. As described above, unlike deletion of *Sav1* or *Nf2*, the deletion of *Mst1* and *Mst2* appears to induce liver damage. Given that most human HCC tumors are thought to develop subsequent to liver damage or chronic inflammation, it will be of interest to test whether or how liver damage and inflammation accelerate HCC development in *Mst1/2*-null mice.

Apoptosis and senescence are the two principal mechanisms of cellular protection against tumorigenesis (Hanahan and Weinberg 2000). Excessive oncogenic signaling such as that mediated by RAS induces senescence (permanent withdrawal from the cell cycle) in otherwise normal cells, and many studies have implicated senescence as a critical tumor suppression mechanism in both human cancers and mouse models (Collado et al. 2005; Braig et al. 2005). Disruption of the senescence pathway often triggers tumor development in cancer models such as those based on RAS activation or loss of the tumor suppressor PTEN (Sarkisian et al. 2007; Chen et al. 2005). Studies have suggested a role for LATS1/2 in the promotion of senescence. LATS2 is thus a target of the oncogenic microRNAs miR372 and miR373, which allow cells to bypass oncogene-induced senescence (Voorhoeve et al. 2006). LATS2 has also been shown to be important for the inhibition of cell proliferation and the induction of senescence markers by the retinoblastoma protein (pRB) (Tschop et al. 2011), and partial ablation of LATS2 suppressed pRB-dependent induction of senescence markers. It will therefore be important to generate Lats1 and Lats2 knockout models in order to test whether senescence mediated by these kinases contributes to their tumor suppressor functions in vivo.

Mice with genetic disruption of the Hippo pathway manifest two key features: expansion of tissue-specific stem or progenitor cell populations, and a hyper-regenerative response and increased cancer incidence after tissue damage. These phenotypes appear to result from YAP activation, given that deletion of one *Yap* allele can prevent their development. These findings highlight the functions of YAP in stem/progenitor cell proliferation and survival and in tissue regeneration. Which target genes of YAP are responsible for these functions? The functional importance of YAP target genes such as those for connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (CYR61), and amphiregulin has been demonstrated in vitro (Zhang et al. 2009, 2011). The importance of these downstream targets and partners of YAP in the Hippo pathway has not been examined with regard to carcinogenesis in vivo, however. Future studies to validate the functions of YAP or TAZ target genes and to identify the transcriptional machinery engaged by these proteins are warranted. Given that inhibition of the epidermal growth factor receptor (EGFR), similar to *Yap* deletion, has been shown to abolish the phenotype associated with NF2 loss (Benhamouche et al. 2010), the mechanism by which the activities of EGFR and YAP might be linked is also worthy of investigation. In addition, the substrates of MST1/2 and LATS1/2 that contribute to the noncanonical functions suggested for these kinases need to be identified and characterized.

Characterization of crosstalk between the Hippo pathway and other cancer-related or developmental pathways will also be important. In vitro studies have implicated YAP and TAZ in diverse developmental pathways such as those mediated by transforming growth factor-β and Smad, by Sonic Hedgehog, or by Wnt and β-catenin (Varelas et al. 2008; Alarcon et al. 2009; Fernandez et al. 2009; Heallen et al. 2011). Systematic analysis of the requirement for YAP or TAZ and for loss of Hippo pathway components in various cancer models will be necessary to examine the role of such cross talk in vivo.

Finally, upstream activating cues for the Hippo pathway in vivo need to be characterized. Studies to date suggest that, unlike other developmental pathways, the Hippo pathway is activated by mechanical cues rather than by soluble factors (Dupont et al. 2011; Schroeder and Halder 2012). This notion, together with the universal effect of disruption of the Hippo pathway on stem or progenitor cell populations, suggests the possible existence of a "physical niche" for such cells. Mechanical forces have been shown to control developmental programs and homeostasis in lower organisms such as *Drosophila* and *Xenopus* (Wozniak and Chen 2009). However, this concept has rarely been tested in higher organisms. Identification of the nature of the niche signals that activate the Hippo pathway in stem or progenitor cells will provide insight into how this pathway restricts the proliferation of these cells.

The Hippo pathway has attracted the attention of many scientists over the course of the last decade. The relevance of this pathway to tumor suppression in vivo has only just begun to emerge. Examination of the importance of the Hippo pathway in more clinically relevant settings, together with refinement and expansion of the mechanistic details of this pathway in mammals, is expected to highlight its conserved central role in tumor suppression from flies to humans over the next decade.

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Chapter 12
Roles of Hippo Signaling During Mouse Embryogenesis

Hiroshi Sasaki

Abstract Embryos undergo dynamic morphological changes during embryogenesis, and elaborate the basic body plan of adults from a single fertilized egg. The Hippo signaling pathway, originally identified as a tumor suppressor signaling pathway in *Drosophila*, is conserved in mice and controls intercellular communication by cell–cell contacts. Recent studies of mouse mutants reveal the roles of Hippo pathway components in the various stages of embryogenesis. Hippo signaling not only regulates cell proliferation and apoptosis but also controls cell fate specification. In this review, I summarize the roles of Hippo signaling during early embryogenesis and discuss the conservation and divergence of the roles and pathways in flies and mice depending upon the developmental stages.

Keywords Mouse development • Preimplantation development • Embryogenesis • Trophectoderm • Notochord • Cell proliferation

12.1 Introduction

12.1.1 Mouse Embryogenesis

The body structure is elaborated from a single fertilized egg during embryonic development. Most morphological changes take place during embryogenesis; in the case of mouse embryos, this occurs during the first 8–9 days after fertilization. Multiple events take place during this period, including cell lineage separation to the future embryo and placenta, body axis formation, and formation and patterning

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_12,

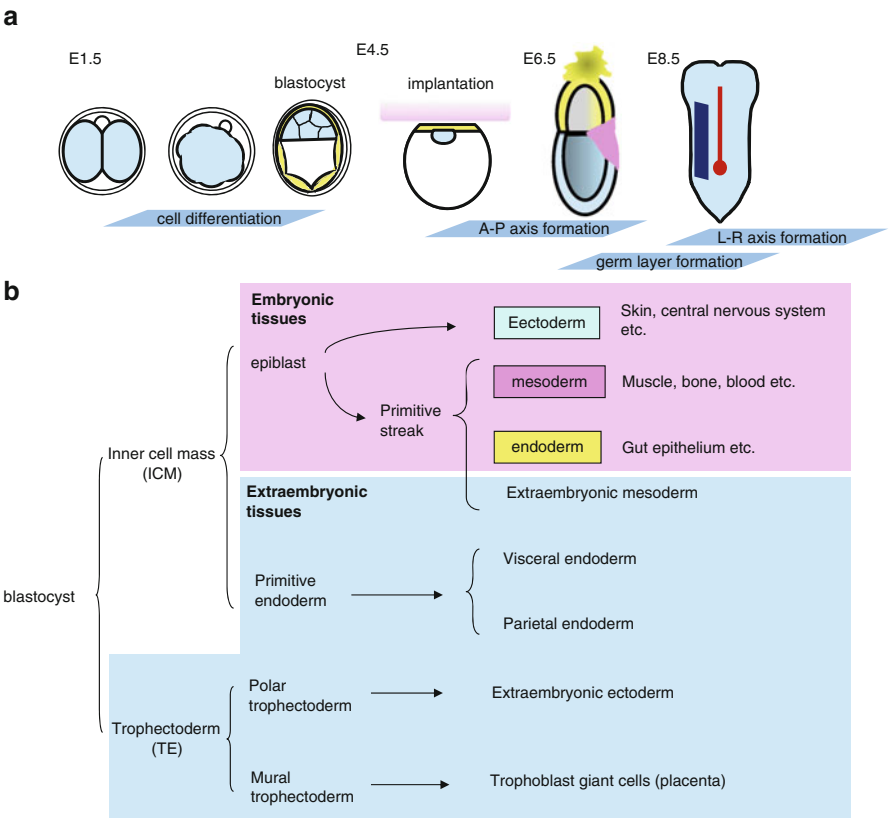

Fig. 12.1 Events and cell lineages in mouse embryogenesis. (**a**) Schematic presentation of major events during mouse embryogenesis. (**b**) Cell lineages in mouse embryos

of the three germ layers (Fig. 12.1). At the cellular level, these processes are controlled by cellular behaviors such as differentiation, proliferation, movement, and death. These processes are controlled and coordinated by intercellular communications, which is thought to be achieved by two mechanisms as follows: one involves long-range communication mediated by secreted signaling molecules and the other occurs by short-range communication mediated by direct cell–cell contacts. Secreted signaling molecules, including Wnt, FGF, BMP, Nodal, and Hedgehog participate in many aspects of embryogenesis. In contrast, relatively little is known about the roles of intercellular communication by direct cell–cell contacts. Identification of the Hippo signaling pathway, which is regulated by cell–cell contact, provides an important clue to address the roles of contact/adhesion-mediated intercellular communication in mouse embryogenesis.

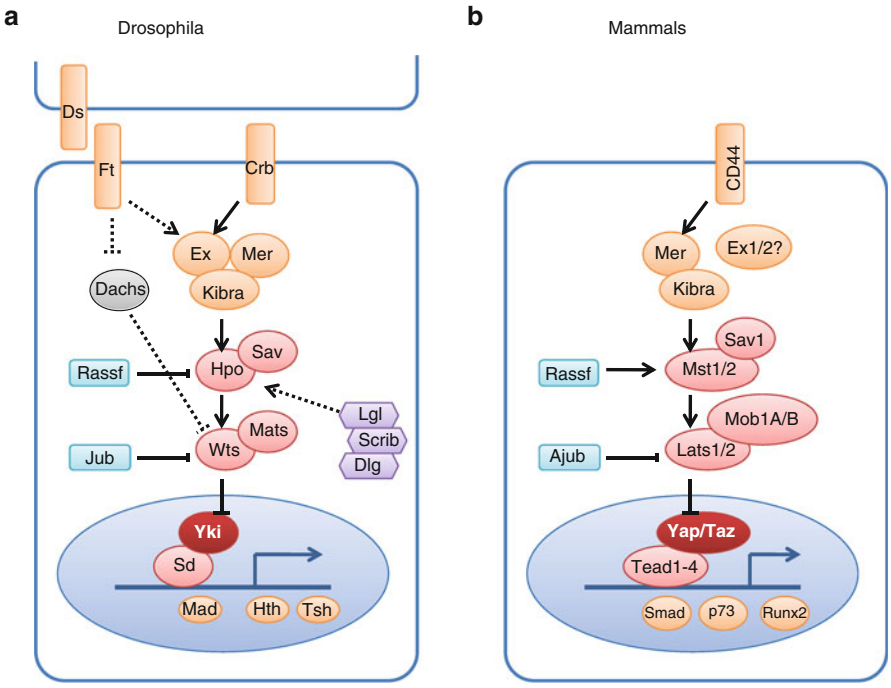

Fig. 12.2 Summary of Hippo signaling pathways in *Drosophila* and mammals. Major components of the Hippo signaling pathways in *Drosophila* (**a**) and mammals (**b**) are shown. The same colors are used to indicate related proteins

12.1.2 Core Components of the Mammalian Hippo Signaling Pathway

The Hippo signaling pathway was originally identified as a tumor suppressor signaling pathway in *Drosophila* (see reviews and references therein Halder and Johnson 2011; Pan 2010; Zhao et al. 2010; Stanger 2012). Genetic screening of *Drosophila* mutants has identified increasing numbers of components of the Hippo signaling pathway (Fig. 12.2a). The core components of the pathway are the protein kinases Hippo (Hpo) and Warts (Wts) and their respective cofactors Salvador (Sav) and Mats (Mob as a tumor suppressor). FERM domain proteins, Merlin (Mer), and Expanded (Ex) are upstream regulators of the kinase cascade, while the coactivator protein Yorkie (Yki) and transcription factors Scalloped (Sd) reside downstream of the cascade. Activation of the Hippo signaling pathway suppresses nuclear accumulation of Yki, which suppresses cell proliferation and promotes apoptosis.

The Hippo pathway is also conserved in mouse (see extensive reviews and references therein Halder and Johnson 2011; Pan 2010; Zhao et al. 2010) (Fig. 12.2b). The mouse genome encodes multiple counterparts of most of the Hippo pathway

components: Mst1/2 (Hpo), Lats1/2 (Wts), Sav1 (Sav), Nf2 (Mer), Frmd6/Willin (Ex), Mob1A/B (Mats), Yap/Taz (Yki), and Tead1/2/3/4 (Sd). Mutations in the genes encoding some of the core components of the mice and human cause cancer (see reviews and references therein Halder and Johnson 2011; Pan 2010; Zhao et al. 2010; Stanger 2012). In cultured mammalian cells, active Hippo signaling suppresses cell proliferation and promotes apoptosis, and this pathway plays central roles in cell density-dependent regulation of cell proliferation, known as contact inhibition (Zhao et al. 2007, 2008; Ota and Sasaki 2008). Besides growth regulation, the mouse mutants in genes encoding Hippo pathway components revealed that Hippo signaling also plays important roles in other cellular processes, including cell differentiation in developing embryos. The roles of Hippo signaling during mouse embryogenesis revealed by genetic analysis are summarized here.

12.2 Cell Fate Control in Preimplantation Mouse Embryos

12.2.1 Preimplantation Mouse Development

During preimplantation development, the mouse conceptus forms a cyst-like structure called blastocyst. A blastocyst consists of an outer epithelial sheet called the trophectoderm (TE) and a cell mass called the inner cell mass (ICM) that is attached to one portion of the inside of the trophectoderm cyst (Figs. 12.1b and 12.3a). The TE is required for the implantation to the endometrium of the uterus and forms the extraembryonic tissues, including the embryonic portion of the placenta and the extraembryonic ectoderm.

The ICM further differentiates into epiblast and primitive endoderm by the time of implantation. The epiblast will form the embryo proper, and the primitive endoderm will form the extraembryonic endoderm. Formation of TE and ICM is the first cell lineage separation in mouse development. Differentiation of TE and ICM is regulated by respective lineage-specific transcription factors (Fig. 12.3a). Cdx2 is a key regulator of TE development (Niwa et al. 2005; Strumpf et al. 2005), and Oct3/4, Nanog, and Sox2 are key regulators of ICM development (Niwa et al. 2005; Nichols et al. 1998; Chambers et al. 2003; Mitsui et al. 2003; Avilion et al. 2003). Cell fates appear to be dependent on cell position within the embryo. At the 8-cell stage, embryos undergo compaction in which E-cadherin-dependent cell–cell adhesion increases and blastomeres acquire apicobasal cell polarity. Up to this stage, all the cells face the outer environment. After the 16-cell stage, some of the cells take the inside positions and lose polarity. By E3.5, the cells taking the outer position express Cdx2 and differentiate into the TE, while the cells taking the inner position express Oct3/4 and differentiate into the ICM (Niwa et al. 2005; Dietrich and Hiiragi 2007).

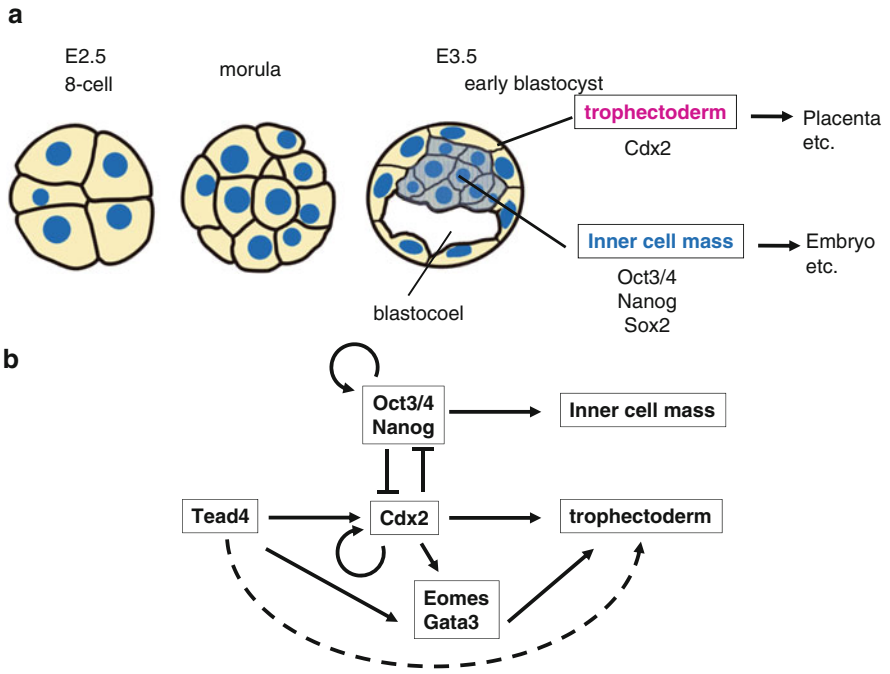

Fig. 12.3 Transcription network controlling preimplantation mouse development. (**a**) Schematic representation of preimplantation development: Outer cells and inner cells will form the trophectoderm and inner cell mass, respectively. (**b**) Network of transcription factors controlling cell fate specification in preimplantation embryos

12.2.2 Tead4 Is Required for TE Fate Specification

The first link between the Hippo pathway and preimplantation mouse development was established by the analysis of mouse mutants of a Hippo pathway transcription factor, Tead4 (Nishioka et al. 2008; Yagi et al. 2007). *Tead4* mutant embryos develop normally up to morula stage and subsequently fail to form the blastocyst and maintain a morula-like appearance. Cell proliferation in the mutant embryos continues normally, but at the later stages, *Tead4* mutant embryos do not express Cdx2 and lack TE. Moreover, all the cells in the embryo express Oct3/4 and become ICM. Therefore, Tead4 is required for TE fate specification (Nishioka et al. 2008; Yagi et al. 2007).

Tead4 is dispensable for ICM lineage or embryo proper (Nishioka et al. 2008; Yagi et al. 2007). Embryonic stem (ES) cells are derived from ICM of the blastocyst. Although *Tead4* is expressed in normal ES cells, *Tead4* mutant ES cells that are able to undergo normal differentiation were established from *Tead4* mutant embryos (Nishioka et al. 2008). Deletion of *Tead4* in cells of the embryonic lineage cells epiblast, the latter which is a pluripotent tissue derived from ICM (Fig. 12.1b), after implantation does not affect the development of embryos, and the resulting

Tead4 mutant mice appear normal (Yagi et al. 2007). Thus, *Tead4* is required only for TE fate specification, and without Tead4, cells are fated to form the alternative, the ICM.

12.2.3 Cell Position-Dependent Hippo Signaling Controls Cell Fates

Tead4 is expressed throughout the embryo but is required only for TE differentiation in the outer cells. How does Tead4 control TE development only in the outside cells? The answer is by Hippo signaling. In preimplantation embryos, Hippo signaling is differentially regulated in a cell position-dependent manner (Fig. 12.4) and is strongly activated in the inside cells, while it is much weaker in the outer cells (Fig. 12.4b). This difference results in a differential subcellular distribution of the coactivator protein Yap (Nishioka et al. 2009) (Fig. 12.4). In the outer cells, weak Hippo signaling results in lower phosphorylation of Yap by Lats and allows nuclear accumulation of Yap. Nuclear Yap increases Tead4 activity. The active Tead4 or Tead4-Yap complex induces the key regulator Cdx2 as well as other TE-specific transcription factors, Gata3 and Eomes. Expression of these transcription factors promotes TE differentiation (Nishioka et al. 2009; Ralston et al. 2010) (Figs. 12.3b, 12.4a, and 12.5).

In the inner cells, strong Hippo signaling phosphorylates Yap via protein kinase Lats1/2 and suppresses nuclear accumulation of Yap. Therefore, Tead4 remains inactive and Cdx2 is not induced (Fig. 12.5). Transcriptional network analysis in ES cells revealed that Cdx2 and Oct4 mutually suppress the expression of the other and activate their own expression (Niwa et al. 2005) (Fig. 12.4b). Similar cross-regulation also occurs between Nanog and Cdx2 (Chen et al. 2009). Therefore, it is likely that the absence of Cdx2 expression allows efficient autoactivation of Oct4 and Nanog in the inner cells, promoting their differentiation into ICM.

12.2.4 Functional Differences Among Tead Proteins

Although three *Tead* genes, *Tead1*, *Tead2*, and *Tead4*, are expressed in preimplantation embryos, only the *Tead4* mutant embryos exhibit abnormalities (Nishioka et al. 2008; Chen et al. 1994; Sawada et al. 2008; Kaneko et al. 2007). Therefore, Tead4 plays a role distinct from that of Tead1/2. This situation is different from the other known functions of Tead. Tead4 regulates transcription in a manner similar to that of the other three Tead proteins (Vassilev et al. 2001). Tead1, Tead2, Tead3, and Tead4 regulate the proliferation of cultured cells in a similar manner (Zhao et al. 2008; Ota and Sasaki 2008).

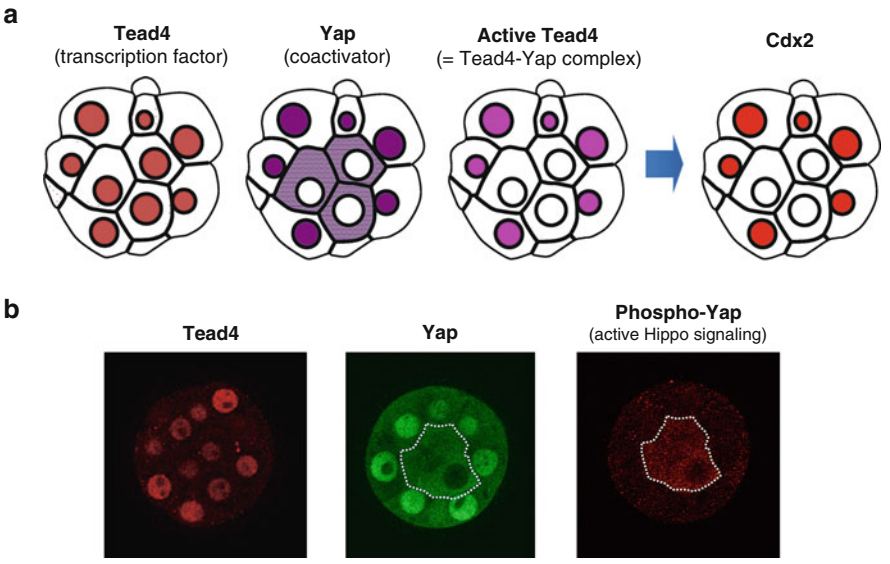

Fig. 12.4 Position-dependent Hippo signaling in preimplantation embryos. (**a**) Schematic presentation of distribution of Tead4, Yap, and the TE regulator, Cdx2. (**b**) Distribution patterns of Tead4 and Yap by immunofluorescent staining: Tead4 is present in all nuclei. The distribution of Yap is position-dependent. Phospho-Yap reflects active Hippo signaling, and this signal is strong in the inner cells. The same embryo was analyzed to detect Yap phosphorylation

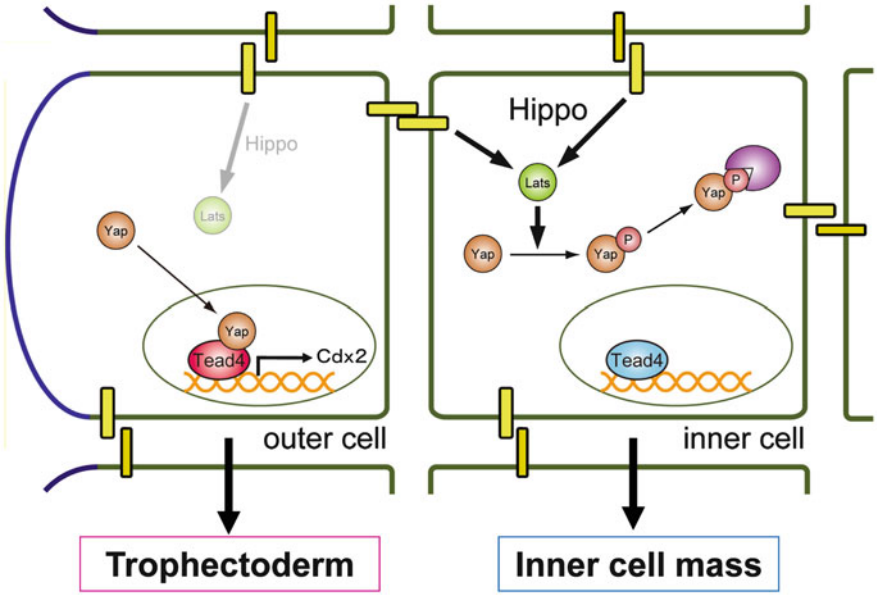

Fig. 12.5 Model of cell fate control by Hippo signaling in preimplantation mouse embryos. Modified from Nishioka et al. (2009)

To the best of my knowledge, the unique functions of Tead4 are unknown. Enforced overexpression of the genetically engineered activator-modified form of Tead4 called Tead4-VP16 (fusion protein comprising the Tead4 DNA binding domain and transcriptional activation domain of herpes simplex virus VP16 protein) in preimplantation embryos induces Cdx2 in the inner cells. Moreover, overexpression of Tead1-VP16 also induces Cdx2 (Nishioka et al. 2009). Thus, it is likely that specificity of Tead4 is not based on its specificity but on the genetic differences that influence the timing or levels of transcription or both.

12.2.5 Hippo Signaling Does Not Control Cell Proliferation in Preimplantation Embryos

In preimplantation embryos, overexpression of Lats2 excludes Yap from all the nuclei and promotes the ICM developmental fate. However, these embryos possess comparable numbers of cells (Nishioka et al. 2009). This observation indicates that the Hippo signal is dispensable for regulation of cell proliferation at this stage. The mechanisms responsible for regulating cell proliferation in preimplantation embryos are unknown.

12.2.6 Possible Mechanisms of Position-Dependent Differential Hippo Signaling

Position-dependent differential Hippo signaling is not a simple correlation. Thus, cell position actually controls Hippo signaling and cell fates (Nishioka et al. 2009). When the cell positions in embryos are manipulated, for example, by dissociation and reaggregation of embryos or isolation of inner cells from early blastocysts, the new outer cells exhibit nuclear accumulation of Yap and become TE irrespective of their original cell position (Nishioka et al. 2009).

The important question is how the Hippo signal is regulated in a position-dependent manner. Activation of Hippo signaling in the inner cells depends on cell–cell contacts. Disruption of E-cadherin-mediated cell–cell adhesion in 16-cell stage embryos by anti-E-cadherin antibody treatment results in decompaction of embryos. The activation of the Hippo pathway is not detectable in such decompacted embryos, and Yap is localized to the nucleus in all cells (Nishioka et al. 2009). One possible explanation for position-dependent Hippo signaling is the differences in the ratio of contact surface to the total cell surfaces. The outer cells have free apical surfaces, whereas the inner cells do not. Thus, such differences would contribute to differences in the activation level of Hippo signaling (Nishioka et al. 2009).

Besides differences in the extent of cell–cell adhesion, outer and inner cells also differ in their cell polarity. The outer cells have apico-basal cell polarity while the

inner cells are apolar. Such differences might also contribute to differences in Hippo signaling. Supporting this hypothesis, polarity-disrupted embryos, in which expression of the cell polarity component Pard6b was knocked-down, showed reduced Cdx2 expression, although the relationship of this effect to Hippo signaling is unknown (Alarcon 2010).

Studies in *Drosophila* and mammalian cells identified the cell polarity regulators, Crumbs, Lgl, and aPKC, as regulators of the Hippo pathway (Chen et al. 2010; Grzeschik et al. 2010; Ling et al. 2010; Robinson et al. 2010; Varelas et al. 2010). However, the situation in preimplantation embryos and *Drosophila* imaginal discs or mammalian cultured cells appears to be the opposite in that Hippo is active in normal polarized cells, and disruption of cell polarity components suppresses Hippo signaling and promotes cell proliferation. In contrast, in preimplantation mouse embryos, Hippo signaling in polarized outer cells is reduced. In contrast, apolar inner cells exhibit strong Hippo signaling (Nishioka et al. 2009). Thus, although polarity likely also plays a role in preimplantation embryos, its underlying mechanisms may be different from those of the epithelium cells.

12.2.7 Divergence of the Hippo Pathway

Rapid accumulation of the information on the roles of individual Hippo components in mouse revealed diversity of the Hippo signaling pathway. For example, Yap is regulated independent of Mst1/2 in mouse embryonic fibroblasts (Song et al. 2010) and independently of Lats1/2 in liver (Zhou et al. 2009). In preimplantation embryos, Yap is regulated by Lats1/2, because Yap accumulates in all nuclei of the *Lats1/2* double mutant embryos (Nishioka et al. 2009). Upstream core components, Mst1/2, Nf2, and Sav1 may not play critical roles, because *Mst1*$^{-/-}$; *Mst2*$^{-/-}$ double mutants; and *Nf2* mutants die during early postimplantation stages (Lu et al. 2010; Song et al. 2010; McClatchey et al. 1997), and *Sav1* mutants die late in gestation (Lee et al. 2008). The alternative pathway that connects cell–cell adhesion and Lats1/2 remains to be elucidated.

12.2.8 Does Tead4 Also Alter Subcellular Distribution in Preimplantation Embryos? A Challenge to the Hippo Signaling Model

Recently, Paul et al. reported that the subcellular distribution of Tead4 is also altered (Home et al. 2012). In mouse trophoblast stem cells (TSCs), Tead4 localizes to the nucleus. In contrast, it resides in the cytoplasm of ES cells. Treatment of ES cells with BMP4 promotes nuclear localization of Tead4. Surprisingly, the differential subcellular localization of Tead4 is also observed in preimplantation mouse embryos

from the 16-cell stage onward. Thus, Tead4 is present in the nuclei of the outer cells and excluded from the nuclei of the inner cells. Moreover, Yap is present in the nuclei of all of the blastomeres. Based on these and other findings, Paul et al. proposed a model in which nuclear transport of Tead4 but not Yap in the outer cells triggers commitment to the trophectoderm lineage (Home et al. 2012).

Their data are distinct from the results of reports by others and are major challenges to the Hippo signaling model described above. Thus, further detailed studies are required before concluding which model is correct. However, it is important to note that we could not reproduce their results. Using the same monoclonal antibody against Tead4, we detected Tead4 in the nuclei of all the blastomeres (both inner and outer cells) at least up to the 32-cell stage (Fig. 12.4b) (Hirate et al. 2012), which are clearly different from the observations of Paul et al. Further, position-dependent differential subcellular distribution of Yap in preimplantation embryos has been independently confirmed by several laboratories (Fig. 12.4) (Hirate et al. 2012).

12.3 Roles of Hippo Pathway Components in Early Postimplantation Embryos

Despite the development of increasing numbers of mouse mutants of genes encoding Hippo pathway components, only a small fraction shows defects at the early postimplantation stage. Further, because of the difficulties in the analysis of the mutant phenotypes at this stage, detailed analyses are not available. In particular, the relationship of the mutant phenotypes to Hippo signaling were not analyzed in some cases (McClatchey et al. 1997; Shimono and Behringer 2003; Xiao et al. 2011). I summarize the mutant phenotypes that are related to or seem to be related to Hippo signaling in a subsequent section.

12.3.1 Tead1 Is Required for Proliferation of the Myocardium

In contrast to its cell fate specification role in preimplantation embryos, the major roles of the Hippo signaling in early postimplantation embryos appear to be regulation of cell proliferation and apoptosis. Tead1's role in cell proliferation was demonstrated by the analysis of Tead1 mutant embryos (Ota and Sasaki 2008). Although Tead1 is expressed widely, early postimplantation Tead1 mutant embryos appeared normal except for hypoplasia of the myocardium. Strong Tead1 expression and clear nuclear Yap signal were observed in the developing myocardium, and Tead1 mutant embryos exhibit severe proliferative defects in the myocardium at E9.5 (Sawada et al. 2008), which results in embryonic death around E11.5 (Chen et al. 1994).

12.3.2 *Tead1/Tead2 Double Mutant Embryos Exhibit Severe Morphological Abnormality Caused by Reduced Proliferation and Apoptosis*

Cell proliferation role of the Hippo pathway is more clearly demonstrated in *Tead1/Tead2* double mutant embryos (Sawada et al. 2008). Tead1 and Tead2 show mostly overlapping expression except in the heart, in which only Tead1 is expressed. The absence of apparent abnormalities in *Tead2*$^{-/-}$ embryos (Sawada et al. 2008; Kaneko et al. 2007) and heart-restricted defects of *Tead1*$^{-/-}$ embryos reflect their functional overlap. At around E7.5, *Tead1/2* double mutants are slightly smaller than normal embryos and lack detectable morphological abnormalities. The embryonic cell populations show reduced cell proliferation and increased apoptosis in all germ layers (Sawada et al. 2008). Because development of this stage does not depend on circulation, these abnormalities reflect the direct roles of Tead1/2. At E8.5, the mutant embryos become significantly smaller than normal embryos and also exhibit severe morphological abnormalities (Fig. 12.6a) and die by E9.5. Despite severe morphological abnormalities at E8.5, germ layer formation and patterning along anteroposterior and mediolateral axes appears relatively normal (Sawada et al. 2008) (Fig. 12.6b). These observations also support the idea that the major roles of Hippo signaling that are mediated by Tead1 and Tead2 are the control of cell proliferation and apoptosis.

In cultured cells, Yap and Tead also induce the epithelial to mesenchymal transition (EMT) (Ota and Sasaki 2008; Overholtzer et al. 2006). Consistent with this observation, *Tead1/2* double mutant embryos start to accumulate cells at the primitive streak at E8.75 (Sawada et al. 2008). Because mesoderm cells are continuously produced from epiblast cells in the primitive streak through the EMT, failure in EMT would cause this phenotype, although detailed molecular analyses are required to substantiate this hypothesis.

Tead1/2 double mutants also suffer from extraembryonic abnormalities, such as disorganized vascularization in the yolk sac. In these mutants, the structural organization of the tissues is disrupted, despite differentiation of the major cell types (endothelial cells and erythroblasts) (Sawada et al. 2008).

12.3.3 *Hippo Signaling Likely Regulates Notochord Differentiation*

The only tissues that show clear defects in gene expression are the node and the notochord, which serve as important embryonic signaling centers present in the midline. The node, which locates at the posterior end of the notochord, contains the notochord progenitor cells and continuously supplies the notochord cells to support posterior extension of the notochord during body axis extension. The node and the notochord are initially formed normally up to E8.25. Subsequently, the notochord becomes discontinuous at E8.5, and both the node and notochord are almost completely

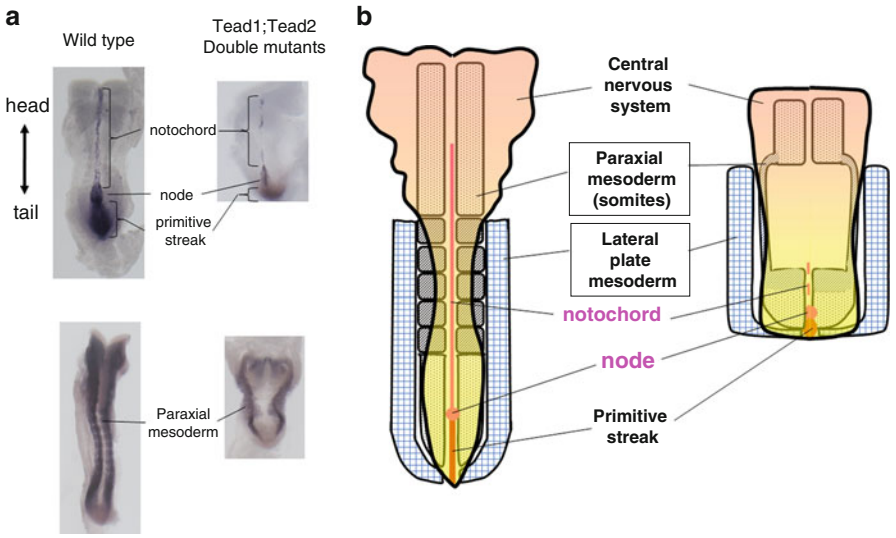

Fig. 12.6 Phenotypes of *Tead1*$^{-/-}$;*Tead2*$^{-/-}$ embryos at E8.5. (**a**) Defects in notochord development (*upper panels*): *Tead1/2* double mutant embryos start to show gradual loss of notochord from E8.5. Expression of Brachyury in the notochord became discontinuous. (*lower panels*) Paraxial mesoderms are formed but slightly shifted laterally. Expression of Foxc1 shifts laterally. (**b**) Summary of mutant phenotypes: Gene expression patterns in the central nervous system (CNS) and mesodermal tissues are summarized. Gradation in the CNS indicates that its patterning along the A-P axis occurs in mutants. The notochord becomes discontinuous and the paraxial mesoderm shifts laterally. Formation and patterning of the endoderm takes place normally (not shown)

lost by E8.75 (Fig. 12.6a, b), which could be explained by either by apoptosis or by loss of identity. Several lines of evidences support the latter hypothesis, although no direct analysis of *Tead1/Tead2* double mutants has been reported. Differentiation of the node/notochord requires the transcription factor Foxa2 (Ang and Rossant 1994; Weinstein et al. 1994), and the enhancer that drives *Foxa2* expression in the node/notochord is directly activated by Tead and Yap (Sawada et al. 2005). At E8.5, the levels of Tead1 and nuclear Yap in the notochordal cells are elevated in comparison with those of the surrounding cells (Ota and Sasaki 2008), indicating weak Hippo signaling and strong Tead1 activity. Thus, in the *Tead1/2* double mutants, lower Tead activity diminishes Foxa2 expression, and differentiation of the node/notochord cells will not be maintained.

12.3.4 Tead1/2 and Yap Cooperates in Embryos

Mouse cells express two Yorkie-related Tead coactivators, Yap1 and Taz. Although these two proteins are structurally closely related, they play distinct roles in mouse

embryogenesis. Yap1 mutant embryos die by E9.5, and their abnormalities closely resemble those of *Tead1/Tead2* double mutants, such as severe morphological abnormalities and smaller sized embryos at E8.5, disorganization of yolk sac vasculature, and notochordal defects (Morin-Kensicki et al. 2006). In contrast, the *Taz* mutant embryos show no apparent abnormalities at this stage (Hossain et al. 2007; Makita et al. 2008). *Tead1/2* and *Yap1* also show clear genetic interaction. At E9.5, $Tead1^{-/-};Tead2^{+/-}$ embryos show clear developmental delay, and these embryos morphologically resemble normal E8.5 embryos. Introduction of a heterozygous mutation of Yap1 into this genetic context ($Tead1^{-/-};Tead2^{+/-};Yap1^{+/-}$) clearly enhances the phenotype. These compound mutant embryos are morphologically indistinguishable from $Tead1^{-/-};Tead2^{-/-}$ mutants. These findings indicate that Tead1/2 and Yap are the major effectors of the Hippo signaling at this stage.

12.3.5 Roles of Other Core Components of the Hippo Pathway

Because of the divergence of the Hippo pathway, it is important to know which core components of the pathway are involved in regulation of Yap and Tead1/2 in early postimplantation embryos. Unfortunately, however, this remains to be determined. None of the Hippo component mutant shows the expected phenotypes, such as overproduction of tissues or larger body size. Instead, defective growth by most embryos is likely caused by secondary effects or by the defects of non-Hippo signaling pathways.

In $Lats2^{-/-}$ embryos, which die around E10.5–E12.5 with global reduction of cell proliferation (McPherson et al. 2004; Yabuta et al. 2007), these abnormalities likely reflect the role of Lats2 as a centrosomal protein that negatively regulates centrosome duplication and not the roles of Hippo signaling components (McPherson et al. 2004; Yabuta et al. 2007). In the case of *Mst1*/2 double mutant embryos, which show slight growth delay at E8.5 and die between E9.5 and E10.5 with reduced body size (Song et al. 2010; Lu et al. 2010), it is likely that defects in yolk sac hematopoiesis secondarily caused global reduction of cell proliferation (Song et al. 2010; Lu et al. 2010). It remains to be elucidated whether Hippo signaling controls hematopoiesis.

The $Nf2^{-/-}$ embryos lack clear extraembryonic ectoderm and develop a smaller ectoplacental cone from E6.5 and die by E8.5 (McClatchey et al. 1997). The mutants lack mesoderm. Chimera analysis revealed that Nf2 is required for extraembryonic ectoderm formation in a cell-autonomous manner, and the absence of this tissue secondarily causes failure of mesoderm induction, which takes place at the boundary of the embryonic and extraembryonic ectoderms (McClatchey et al. 1997). It remains to be elucidated whether development of the extraembryonic ectoderm is regulated by Hippo signaling. If so, then Hippo signaling also must play roles in specification of extraembryonic ectoderm fate or self-renewal, or both, of the TSCs as well as initial TE fate specification in preimplantation embryos.

12.4 Perspective

Recent development of mutant mice harboring mutations in genes encoding Hippo pathway components is revealing the roles of Hippo signaling in mouse embryogenesis. Because of the divergence of the signaling pathway between model organisms, it is important to clarify the mechanisms by which Hippo signaling is regulated in embryos. Currently, information about Hippo signaling in early postimplantation embryos is very limited. It remains unclear whether Hippo signaling plays critical roles in negative regulation of cell proliferation at this stage. Little is known about the cell-autonomous functions of each Hippo pathway component. Future studies using combinations of conditional mutants and appropriate *Cre* lines might clarify the roles and regulatory mechanisms of Hippo signaling during embryogenesis.

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Chapter 13
Hippo Signaling and Stem Cells

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Abstract The normal growth and development of an organ is dependent on the precise balance of stem cell self-renewal and differentiation. Slightest aberrations in signals stem cells receive can cause growth abnormalities and cancer. Emerging data suggest that the highly conserved Hippo signaling pathway can directly regulate stem cell proliferation and maintenance to control organ size. Furthermore, deregulation of the pathway promotes cancer stem cell-like properties and leads to tumor formation. Together, these findings implicate that the Hippo pathway modulates the dynamic activity of stem cells in tissue repair, regeneration, and development. Here, we summarize the latest findings that establish the role of Hippo pathway in stem cell biology.

Keywords Stem cell • Cancer • Hippo pathway • Organ size • Cancer stem cell

13.1 Introduction

Stem cells are unique cell types that can differentiate to produce diverse cells in the body. During development, the regulation of stem cell number is key in determining the final size of an organ (Depaepe et al. 2005; Stanger et al. 2007). For instance, depletion of forebrain neural progenitors in developing mice leads to reduced cortical

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_13,

size. Conversely, increasing the progenitor number causes increased cortical size leading to exencephalic forebrain overgrowth (Depaepe et al. 2005). A similar phenomenon has been described in the mouse pancreas where the size of the progenitor cell pool, set aside in the developing pancreatic bud, determines the size of the organ (Stanger et al. 2007). Clearly, proper regulation of organ size requires that stem cells integrate the surrounding environmental cues and respond appropriately; however the mechanism by which this occurs is poorly understood.

The Hippo Signaling pathway has emerged as a key regulator of organ size control. The pathway was first characterized in *Drosophila melanogaster* where deregulation of the pathway was shown to cause a strong overgrowth phenotype (Justice et al. 1995). The *Drosophila* Hippo signaling pathway is highly conserved through evolution having mammalian orthologues of all pathway components. Consistent with its role in organ size regulation in *Drosophila*, the mammalian Hippo pathway (Fig. 13.1) is also linked to organ size regulation mainly by controlling cell proliferation and apoptosis. The core mammalian Hippo pathway consists of the STE20 family kinases, MST1 and MST2 together with their regulatory protein SAV1. MST1/2 form an activated complex when bound to SAV1 (Pan 2010; Zhao et al. 2011). Interaction with the RASSF family of proteins also activates these kinases (Khokhlatchev et al. 2002; Oh et al. 2006) which then phosphorylate the NDR family kinases LATS1 and LATS2 (Dong et al. 2007; Chan et al. 2005; Hirabayashi et al. 2008). MST1/2 also phosphorylate the MOB1 complex (MOBKL1A and MOBKL1B) to enhance the interaction with the LATS1/2 kinases. MOB1 acts as a regulator of LATS1/2 activity (Praskova et al. 2008).

LATS1/2 kinases phosphorylate the paralogous transcriptional coregulators Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) (Hao et al. 2008). Phosphorylation of YAP/TAZ promotes their interaction with the 14-3-3 family members thereby keeping them localized in the cytoplasm (Dong et al. 2007; Hao et al. 2008; Oh and Irvine 2008; Zhao et al. 2007; Lei et al. 2008; Oka et al. 2008). The binding of 14-3-3 is primarily mediated by Ser89 residue in human TAZ (Ser 87 in mouse TAZ) and Ser127 in human YAP (Ser112 in mouse YAP) (Kanai et al. 2000; Basu et al. 2003). Low LATS1/2 activity allows the unphosphorylated YAP/TAZ to localize into the nucleus and execute their transcriptional functions. In the nucleus, YAP/TAZ can act as coactivators for several transcription factors (Mauviel et al. 2012) (Sudol and Harvey 2010) although preferentially coregulating the members of the TEAD family of transcription factors (Zhao et al. 2008; Schlegelmilch et al. 2011). There are four members of the mammalian TEAD family transcription factors that share the same TEA DNA-binding domain (Kaneko and DePamphilis 1998; Jacquemin et al. 1998). At least one member of the TEAD transcription factor family is expressed in almost all adult tissues (Jacquemin et al. 1998; Kaneko et al. 1997). The upstream regulators of the pathway, potentially sensing the environmental cues, are not well established. The Neurofibromatosis2 gene product NF2 (also known as Merlin) is the only upstream component that is functionally validated in vivo (Hamaratoglu et al. 2006; Zhang et al. 2010). However, it is still unclear how the cytoskeleton and membrane-associated NF2 protein signals to the MST kinases. A more thorough description of these and other pathway components can be found in other chapters of this book.

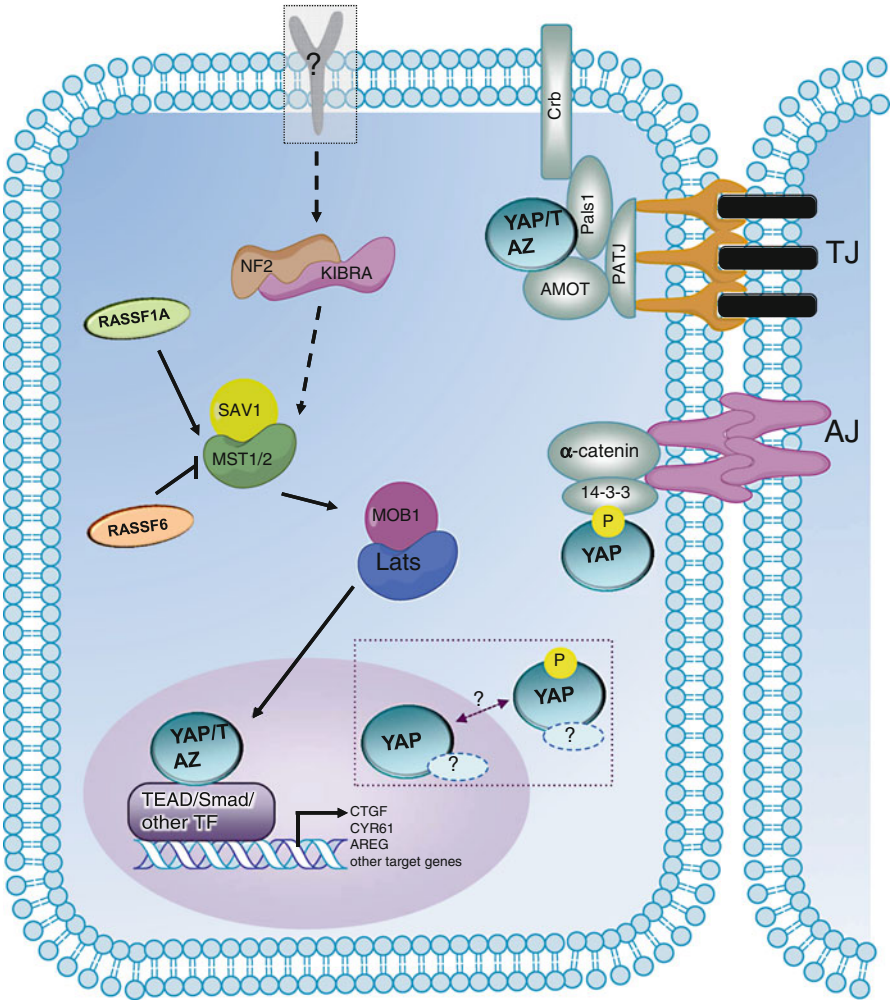

Fig. 13.1 The mammalian Hippo signaling pathway. Blunted or arrowed end depicts inhibition and activation, respectively. *Dashed line* depicts unknown mechanisms *solid line* depicts known interactions

13.2 Hippo Signaling in Adult Stem and Progenitor Cells

13.2.1 Liver Progenitor Cells

The first characterization of the roles of YAP in vivo demonstrated a critical importance of the Hippo pathway in controlling liver size (Dong et al. 2007; Camargo et al. 2007). The regenerative capacity of the liver is remarkable and of significant clinical importance. Following partial hepatectomy, the regenerative process in the

liver is mostly mediated by the cell cycle reentry and proliferation of the terminally differentiated hepatocytes. However, in some cases where the proliferation of hepatocytes is suppressed, regeneration of the liver can occur via expansion and proliferation of a putative periportal liver stem cell population, the oval cells (Avruch et al. 2011).

Camargo et al. generated transgenic mice carrying a doxycycline (Dox)-inducible YAP allele targeted to the collagen1a1 locus. The YAP construct used in these studies carries a mutation in residue 127 (Ser->Ala) mimicking a constitutively active form of YAP (Camargo et al. 2007). Using a tetracycline transactivator (rtTA) under the control of the hepatocyte-specific liver activator protein (LAP) they created a model in which YAPS127A expression could be temporally and spatially controlled in hepatocytes upon Dox induction. Remarkably, administration of Dox to postnatal animals resulted in a fourfold in total liver mass (Camargo et al. 2007). It was shown that this overgrowth resulted from proliferation of mature hepatocytes, and not oval-like cells, that were also less resistant to Fas-mediated apoptosis (Camargo et al. 2007). Another independent study described a similar YAP-induced liver overgrowth phenotype using a transgenic mouse carrying the wild-type human YAP cDNA also under the tetracycline-response element and using an rtTA driver under control of ApoE promoter (Dong et al. 2007). In both studies, the overgrowth phenotype could be completely reversed upon withdrawal of Dox to discontinue YAP expression (Dong et al. 2007; Camargo et al. 2007). Activating YAP for longer periods of time led to the development of hepatocellular carcinoma in adult livers (Dong et al. 2007; Camargo et al. 2007). The YAP overexpression phenotype in the liver can be fully rescued by expressing a dominant-negative form of TEAD2 that lacks its DNA-binding domain (Liu-Chittenden et al. 2012). This rescue of the increased liver size suggests that YAP's proliferative function in the liver is dependent on the TEAD transcription factors (Liu-Chittenden et al. 2012).

The inducible deletion of upstream factors of the pathway such as SAV1, MST1/2, and NF2 has confirmed their role in regulating YAP in vivo. Livers carrying mutations in these genes display elevated levels of nuclear localized YAP and also display dramatic increases in liver size. And in similar fashion as the YAP overexpression experiments, tumors develop in these models, which resemble both hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) malignancies (Zhang et al. 2010; Zhou et al. 2009; Song et al. 2010; Lee et al. 2010; Lu et al. 2010). Intriguingly, the cellular phenotype in these mutants has been described as an oval cell-like expansion, and the fact that tumors display mixed biliary and hepatocytic morphologies supports the hypothesis that a bipotential progenitor was expanded. It is still a bit unclear why the cellular phenotype differs between the YAP overexpression models and the other mutants. It will be essential to perform hepatocyte or oval cell-specific gene manipulations to fully understand the cell types that are endogenously more sensitive to Hippo signaling requirements.

Intriguingly, the connection of NF2 with YAP was disputed by a study that credited the NF2 mutant phenotype to aberrant activity of the epidermal growth factor receptor (EGFR) rather than to the Hippo pathway (Benhamouche et al. 2010). Nevertheless, the rescue of the NF2 mutant phenotype by deletion of only one allele

of YAP constitutes strong functional evidence that the main tumor suppressive mechanism of NF2 is through YAP inactivation (Zhang et al. 2010). Collectively, these studies suggest that the Hippo pathway plays a crucial role in maintaining the quiescence of the postnatal liver and that its misregulation can lead to overgrowth and tumorigenesis. A big question in the field is the nature of the transcriptional program that YAP activates, as there is no functional evidence that the described targets are important for the YAP-driven phenotypes.

13.2.2 Intestinal Stem Cells

The intestinal epithelium renews continuously to maintain tissue homeostasis, turning over entirely every 4–5 days. The rigorous process of repair and renewal relies on the continuous proliferation of the intestinal stem cells (ISCs) located at the base of the intestinal crypt. Using an inducible mouse model that expressed YAP-S127A ubiquitously, it was first reported that YAP activation caused a reversible expansion of undifferentiated progenitor-like cells in the small intestine. This population expanded from the bottom of the crypt upwards and ended up replacing the mature cell types along the intestinal villi (Camargo et al. 2007). Within 5 days of YAP activation, the entire intestinal epithelium became highly proliferative and showed absence of differentiated enterocytes, mature goblet cells, and Paneth cells. Upon interruption of YAP expression by Dox withdrawal, the intestinal progenitors were able to resume their differentiation program, indicating a requirement for YAP expression in the progenitor expansion phenotype (Camargo et al. 2007). Similarly, Mst1/2-deficient intestines exhibit marked expansion of an undifferentiated progenitor population accompanied by a dramatic decrease of secretory lineages. In this model the enterocytes were better preserved compared to the Yap overexpression model (Zhou et al. 2011). It was further reported that ablation of a single YAP allele can suppress the hyperproliferative phenotype in MST1/2 deleted intestines. Similarly, deletion of Sav in the intestine leads to a hyperproliferative phenotype in the colon with a very long latency (Fre et al. 2009). This phenotype was also fully rescued by the concomitant loss of YAP (Fre et al. 2009).

Intriguingly, loss of YAP in the intestine does not lead to any major phenotype during development or normal homeostasis of the intestine (Fre et al. 2009). Given that YAP is normally expressed in the crypts of the intestine and presents a robust nuclear localization there (Zhang et al. 2010), it was quite surprising that YAP loss did not lead to a phenotype in intestinal development or renewal. It remains to be seen whether TAZ plays a compensatory role or simply YAP is not necessary for normal homeostasis. A role for YAP in tissue repair was demonstrated following challenging with the colitis-inducing agent dextran sulfate sodium (DSS). YAP-deficient mice were not able to recover from a DSS challenge due to an inability to jumpstart a proliferative response to the injury (Zhang et al. 2010). It will be interesting to see if this phenotype is observed following other regimes that induce regeneration such as irradiation or chemotherapy-induced intestinal injury. An

intriguing thought is that YAP is part of a regeneration-specific molecular response in the intestine.

Recently, work has begun to provide insight in to the mechanisms used by YAP to control intestinal progenitor biology. YAPS127A-induced expansion of the intestinal progenitor compartment was partially credited to Notch pathway activation given the upregulation of the Notch target gene Hes1 in mutant intestines. Additionally, short-term chemical inhibition of the Notch pathway using γ-secretase inhibitors reduced the dysplastic phenotype in intestine (Camargo et al. 2007). This will be better addressed by genetic studies that utilize mutants for the Notch pathway. MST1/2 null intestine also display strong activation of the Wnt and the Notch signaling pathway (Zhou et al. 2011). Interestingly, the mRNA levels of a Notch ligand, Jagged 1, was significantly increased while the mRNA level of the Notch receptor, Notch1, remained unchanged in MST1/2 mutant intestine. Since Jagged 1 is not known to be a direct target of YAP, but is a direct target of the Wnt pathway, upregulation of the Notch Pathway was hypothesized to be likely consequence of YAP-induced Wnt activation (Zhou et al. 2011). Given these potential functional interactions, several studies have explored the mechanism of cross talk between these pathways in the intestine. It is known that the Wnt and Notch signaling pathway synergistically play an important role in progenitor maintenance and proliferation (Fre et al. 2009). In 2010, a study demonstrated that the Hippo pathway restricts Wnt signaling by sequestering Dishevelled (DVL) in the cytoplasm by promoting its interaction with cytoplasmic TAZ (Varelas et al. 2010a). More precisely, TAZ controls Wnt signaling by inhibiting the CK1delta/epsilon-mediated phosphorylation of DVL (Varelas et al. 2010a). The unphosphorylated DVL induces activation of the Axin-APC-GSK3 destruction complex that leads to the degradation of the Wnt pathway transcriptional regulator, β-catenin (Clevers 2006; Nusse 2005). It has also been shown that cytoplasmic YAP could directly bind to β-catenin to mediate its cytoplasmic retention (Imajo et al. 2012). While these observations indicate a potential Wnt repressive role for cytoplasmic YAP, it is still unclear how nuclear YAP might activate progenitor proliferation. Still these studies indicate that the Hippo pathway plays a crucial role in the tight control of intestinal proliferation and differentiation partially or entirely via other signaling pathways.

13.2.3 Neural Progenitor Cells

Multipotent neural progenitor cells that generate the central nervous system reside along the ventricular zone in the developing vertebrate neural tube (Merkle and Alvarez-Buylla 2006). In 2008, it was reported for the first time that the Hippo pathway could regulate the neural stem cell population. The overexpression of YAP in the chick neural tube resulted in an increased number of neural progenitor cells (Cao et al. 2008). Inhibiting upstream Hippo kinases, LATS1/2 and MST2, also led to an increase in the neural progenitor pool. This result demonstrated that the MST-LATS kinase cascade was important in mediating the YAP function in neural stem

cells (Cao et al. 2008). Akin to the findings in the intestine, Hippo inactivation or YAP expression led to increased self-renewal and reduced differentiation of neural progenitor cells. The proliferative effect of YAP could be rescued by disrupting the YAP–TEAD interaction, suggesting TEAD transcription factors mediate YAP activity in neural stem cells (Cao et al. 2008).

Cerebellar granule neuron precursors (CGNPs) are speculated to be the cell of origin for some medulloblastomas (Provias and Becker 1996). Sonic hedgehog (Shh) pathway regulates the proliferation of CGNPs and its aberrant activation can lead to the formation of medulloblastoma (Dahmane and Ruiz i Altaba 1999; Raffel et al. 1997; Reifenberger et al. 1998). Fernandez et al. indicated that YAP was highly expressed in a subset of medulloblastomas that were driven by Shh deregulation (Fernandez et al. 2009). Shh induces YAP expression and promotes YAP nuclear localization in CGNPs leading to increased proliferation (Fernandez et al. 2009). Ectopic expression of YAP in CGNPs grown without Shh in culture demonstrated higher mRNA level of known Shh effector, Gli2. Further, ChIP analysis revealed that YAP binds two of the four putative TEAD-binding sites on Gli2 promoter region. A possible mechanism of YAP-mediated CGNP proliferation could be via Gli2 induction, which then activates other downstream mediators of Shh signaling in CGNPs. In mouse medulloblastomas, YAP localizes to cells in the perivascular niche (PVN) that are proposed to be the tumor-repopulating cells (Fernandez et al. 2009). These YAP expressing PVN cells are resistant to irradiation and contribute to tumor regrowth (Fernandez et al. 2009). These findings indicate that YAP is an effector of the Shh pathway and a potential therapeutic target for medulloblastoma.

Recently, Li et al. (2012) reported that Notch activation leads to symmetric neural stem cell division by studying a new transgenic mouse model in which activated form of NOTCH1 receptor can be conditionally expressed in maturing neuroepithelium (Li et al. 2012). ChIP-seq and transcriptome analysis revealed that the transcription factors of the Hippo, Wnt, and Shh pathways are direct targets of the Notch pathway in neural stem cells in vivo (Li et al. 2012). Furthermore, Li et al. (2012) showed that YAP is selectively expressed in the stem cell compartment in the developing forebrain. Ectopic YAP expression rescues the effect of Notch inhibition suggesting that YAP is an effector of the Notch pathway in neural stem cells. The existing studies in neural progenitor cells implicate the complicated cross talk between the Notch, Shh, Hippo, and other pathways that may be required to maintain the proper regulation of the NSCs. Future studies should validate these observations in transgenic mice with stem cell-specific deletions of Hippo signaling molecules.

13.2.4 Epidermal Progenitor Cells

Similar to the intestinal epithelium, the skin regenerates continuously and relies on the proper balance between quiescence and differentiation of the epidermal progenitor cells that reside in the basal layer to maintain homeostasis. The development of the mammalian skin starts as a single-layered multipotent embryonic progenitors that

differentiate to generate epidermis, sebaceous glands, and hair follicles (Fuchs 2007). On embryonic day 14, mouse epidermis exists as single-layered basal epidermal progenitors that express nuclear YAP (Zhang et al. 2011). The nuclear expression of YAP progressively declines as the proliferative capacity of the basal epidermal progenitor is reduced. By postnatal day 11, nuclear expression of YAP in basal epidermal cells is restricted to very few cells in the basal cell layer (Zhang et al. 2011).

Conditional deletion of YAP in epidermal progenitor cells of developing mice causes loss of progenitor cells resulting in an overall thinning of the skin and an absence of epidermal tissue in the distal part of the limbs, eyes, and ears in E18.5 mouse embryos (Schlegelmilch et al. 2011). Overexpressing constitutively active YAP in Keratin 14 expressing epidermal cells results in the expansion of the interfollicular stem cell compartment and abnormal thickening of the skin eventually leading to squamous cell carcinoma-like tumors (Schlegelmilch et al. 2011; Zhang et al. 2011). Developmental ablation of SAV1 in mice leads to similar hyperproliferation of skin epithelial progenitors in a manner very reminiscent of the YAP activation model (Lee et al. 2008). However, epidermal-specific ablation of MST1/2 and knockdown of LATS1/2 in HaCaT keratinocytes showed no effect on YAP phosphorylation suggesting that YAP activity is regulated by alternative mechanisms in this cellular context (Schlegelmilch et al. 2011). Two independent studies have shown that an adherens junction component, α-catenin, acts as an upstream negative regulator of YAP and sequesters YAP in the cytoplasm (Schlegelmilch et al. 2011; Silvis et al. 2011). The adherens junctions (AJs) have been speculated to act as molecular biosensors of cell density (Silvis et al. 2011; Lien et al. 2006a; Lien et al. 2006b). Additionally, several studies have demonstrated the association of YAP and TAZ with polarity proteins and cell–cell contact-regulating proteins (Robinson et al. 2010; Ling et al. 2010; Chen et al. 2010; Grzeschik et al. 2010; Skouloudaki et al. 2009; Varelas et al. 2010b; Doggett et al. 2011; Kim et al. 2011). The data linking YAP and α-catenin establishes YAP as a key component of a "crowd control" molecular circuitry in the epidermis.

13.2.5 Cardiac and Skeletal Muscle Progenitor Cells

Maintaining an optimal size is essential for any organ in the body but it becomes even more crucial in cardiac development. The heart must be large enough to pump sufficient volume of blood but not so large that it blocks the outflow of blood from the left ventricle. A recent study inactivated the Hippo pathway by knocking out the upstream regulator, SAV1, in developing mouse hearts; and observed that these mutant embryos had larger hearts with elevated cardiomyocyte proliferation (Heallen et al. 2011). Gene interaction studies uncovered a nuclear interaction between YAP and β-catenin, a well-known promoter of growth in the heart. Loss of β-catenin in SAV1 conditional knockout mouse hearts rescued the overgrowth phenotype caused by Hippo inactivation, implicating that the Hippo pathway restrains cardiomyocyte proliferation and heart size by inhibiting Wnt signaling (Heallen et al. 2011).

Another recent study showed that conditional deletion of YAP in cardiac progenitor cells during cardiogenesis leads to lethal embryonic myocardial hypoplasia while overexpressing constitutively active YAP leads to cardiomyocyte proliferation (Xin et al. 2011). The pro-proliferative activity of YAP in the heart is mediated by its interaction with the TEAD transcription factors (Xin et al. 2011; von Gise et al. 2012). It was suggested that constitutively active YAP promotes cardiomyocyte proliferation and cardiomegaly by coupling with insulin-like growth factor (IGF) and Wnt signaling (Xin et al. 2011). YAP drives cardiac proliferation by activating the IGF pathway followed by glycogen synthase kinase 3b inactivation, which in turn inhibits the β-catenin destruction complex resulting in increased levels of β-catenin. Therefore, YAP promotes cardiomyocyte proliferation by intensifying Wnt signaling directly by interacting with nuclear β-catenin or indirectly via the IGF pathway (Heallen et al. 2011; Xin et al. 2011; von Gise et al. 2012; Shiojima and Walsh 2006; Matsui et al. 2008).

A role for the Hippo pathway in skeletal muscle was also recently reported. Yap overexpression in C2C12 myoblasts and primary mouse muscle stem cells blocks the progression of the myoblasts through the myogenic program and preserves the progenitor-like and proliferative states (Watt et al. 2010; Judson et al. 2012). Interestingly, TAZ, despite the high level of sequence identity with YAP, was shown to promote differentiation of myoblasts by promoting Myod1 activity (Jeong et al. 2010). This opposite effect of the two Hippo pathway effectors, YAP and TAZ, on muscle progenitor fate is a good illustration of the complexity and context-specificity associated with Hippo pathway activation or inhibition and the resulting transcriptional response. Obviously, further studies done in transgenic mice are needed to conclusively determine the role of Hippo signaling in skeletal muscle biology.

13.3 Hippo in Embryonic and Induced Pluripotent Stem Cells

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst stage of the preimplantation embryo. TAZ and YAP null embryos do not survive past the morula stage because nuclear localization of Yap/Taz in the outside cells of the preimplantation embryo is required to form the trophoectoderm (Nishioka et al. 2009). Coordination of multiple signaling pathways is crucial in maintaining the balance between differentiation and self-renewal of ESCs. Human embryonic stem cells (hESCs) depend on the TGF-β/Activin signaling, BMP signaling, and FGF signaling for self-renewal (Biswas and Hutchins 2007; Darr and Benvenisty 2006; Xiao et al. 2006). The TGFβ/Activin/Nodal signaling is transduced by the SMAD2/3 complex (James et al. 2005; Vallier et al. 2005). Varelas et al. showed that TAZ is responsible for shuttling the SMAD2/3 complex in and out of nucleus in response to the TGFβ signaling (Varelas et al. 2008). Therefore, knockdown of TAZ in hESCs leads to disruption of TGFβ signaling leading to loss of its pluripotent state. Mouse embryonic stem cells (mESCs) rely on the cytokine leukemia inhibitor factor (LIF) signaling and BMP signaling to maintain their

stemness (Evans 2011; Chambers and Smith 2004). The BMP pathway signaling is propagated via the SMAD1/5/8 signal transducer complex (Ying et al. 2003). Chip analysis revealed that YAP and SMAD1 are bound to the BMP-responsive Id gene family during active transcription in response to BMP signaling. This piece of evidence suggests that YAP associates with SMAD1 to enhance BMP-mediated transcription required for hESC maintenance (Alarcon et al. 2009).

Supporting a role for YAP and TEADs in pluripotency, Tamm et al. found that YAP and TEAD2 are highly expressed in ESCs and downregulated when cells undergo differentiation (Tamm et al.). Moreover, YAP/TEAD2 could activate the expression of the ESC master transcriptional regulators Oct4 and Nanog, and TEAD function inhibition resulted in differentiation towards the endoderm lineage (Tamm et al.). Another group reported similar findings and provided additional evidence for the role of YAP in pluripotency using induced pluripotent stem cells (iPSCs) (Lian et al. 2010). The seminal findings of Takahashi et al. demonstrated that mouse somatic cells can be reprogrammed into iPSCs by inducing the activity of four transcription factors, Sox2, Oct3/4, c-Myc, and KLF4. YAP is activated during the reprogramming of human embryonic fibroblasts into iPSCs and addition of YAP to Sox2, Oct4, and KLF4 transcription factors infection in mouse embryonic fibroblasts increases the iPSC reprogramming efficiency (Lian et al. 2010). In conclusion, YAP and TEAD proteins seem to be critical factors for the maintenance of pluripotent properties of both ESCs and iPSCs. Further studies should fully evaluate the role of YAP, TAZ, and other Hippo pathway components using full genetic loss-of-function alleles and should aim to determine how YAP/TEAD interact with known stem cell signaling and transcriptional networks.

13.4 Hippo in Cancer Stem Cells

Cancer stem cells (CSCs) are thought to be the tumorigenic cell types in cancer that have stem cell-like properties. These cells constitute only a fraction of the tumor cells, but they have the ability to self-renew and differentiate into other tumor cell types (Visvader and Lindeman 2008). These cells have been shown to be resistant to chemotherapy, and are thought to be responsible for cancer relapse. High-grade tumors are characterized by a higher accumulation of these CSCs (Pece et al. 2010). A screen done in 993 primary human breast tumors revealed that the Hippo signaling gene signature was overrepresented in high-grade (G3) tumors implicating elevated TAZ/YAP activity in high-grade tumors (Cordenonsi et al. 2011).

Studies done using MCF10A-T1k cells (MII) and MCF10A-CA1a cells (MIV) shed light on the role of TAZ in breast cancer cells. Upon injection into mice, the MII cells generate low-grade tumors, whereas the MIV cells generate high-grade tumors. Interestingly, TAZ was highly expressed in MIV cells as compared to the MII cells, while YAP level was comparable. Knocking down endogenous TAZ in MIV cells significantly reduced its potential to produce primary and secondary tumors and caused a 20-fold reduction in the tumor-initiating cells (Cordenonsi

et al. 2011). FACS sorting the MII cells according to CD44 and CD24 expression revealed that the $CD44^{high}/CD24^{low}$ population, that has CSC-like properties, expresses higher levels of TAZ. Knocking down TAZ in $CD44^{high}/CD24^{low}$ cell population led to reduced self-renewal properties (Cordenonsi et al. 2011). Overexpressing constitutively active TAZ in this cell population caused increased cell proliferation, higher ability to form primary, secondary, and tertiary tumors that are more resistant to chemotherapeutic drugs. The TAZ overexpressed MII cells produce invasive carcinoma similar to the ones from MIV cells (Cordenonsi et al. 2011). Collectively, the findings indicate that TAZ is required to sustain self-renewal capacity and tumorigenic potential in breast cancer cells.

Another study done in glioblastomas (GBM) showed that nuclear TAZ is highly expressed in high-grade GBMs. Tumors enriched with a neural development proneural (PN) gene signature display a higher survival rate when compared to tumors with a high mesenchymal (MES) gene expression signature (Bhat et al. 2011). TAZ was hypermethylated in the PN group of tumors compared to the MES group, where at the YAP methylation status was comparable. Silencing TAZ in the MES glioma stem cells (GSCs) led to decreased invasive ability, self-renewal, and tumor-initiating capacity (Bhat et al. 2011). Overexpression of TAZ in PN GSCs induced expression of the MES marker, thus driving aberrant osteoblastic and chondrocytic differentiation. The high-grade breast cancer tumors and glioblastoma tumors present a considerable clinical challenge since they show resistance to chemotherapy as well as radiation. TAZ, thus presents as a novel molecular target for treating these aggressive tumors.

13.5 Conclusions

Since its discovery in the fruit fly, much progress has been made in the Hippo signaling field and it is now widely accepted that this pathway and its effector, YAP, play critical roles in mammalian stem and progenitor cells and growth control. Nonetheless, important questions regarding the identity and physiological relevance of upstream Hippo modulators are yet to be answered. As many different components are identified in cell culture experiments, it is important to validate these observations in a physiological context. Recent data implicating cell polarity and adhesion and mechanotransduction as inputs that regulate Hippo kinase activity provide exciting avenues to explore. Another area that is bound to provide important insight into the biology of size control is the definition of the mechanisms by which Hippo and YAP cross talk with other developmental signaling pathways. Current evidence suggests an important relationship between Hippo, Wnt, Hedgehog, Notch, and BMP pathways (Camargo et al. 2007; Zhou et al. 2011; Fernandez et al. 2009; Li et al. 2012; Varelas et al. 2008; Alarcon et al. 2009). These relationships need to be further validated using in vivo animal models and better-defined biochemically.

While the importance of the Hippo pathway in some stem cell populations is well documented, its role in other stem cell populations still remains unknown.

Interestingly, overexpression of YAP in the hematopoietic system revealed no changes in the distribution of the hematopoetic lineages and number/function of hematopoetic stem cells (Jansson and Larsson 2012). Deletion of YAP in the hematopoietic system also does not lead to any major defects (F. Camargo, unpublished data). It is quite intriguing how a pathway that is such a potent regulator of proliferation in epithelial tissues, seems to have absolutely no effect on the proliferation of blood progenitors, even when YAP is overexpressed. It will be interesting to explore what molecular features of blood cells or other tissue-specific progenitors make them insensitive or more sensitive to YAP and TEAD activity.

Organ size is one of the least understood questions in developmental biology. It is now fair to speculate that proper tissue size is achieved through a combination of morphogenic signaling, patterning cues, spatial control of YAP/TAZ localization by cell–cell contact, and mechanical cues dictated by tissue architecture. Further investigation of these processes and how they ultimately converge on Hippo signaling will likely provide insight into molecular mechanisms that regulate development, stem/progenitor renewal, regeneration, and cancer biology.

Acknowledgements We apologize to those investigators whose work we could not cite due to limited space. We thank Annie M. Tremblay, Evan Barry, and Morvarid Mohseni for providing feedback and proofreading this manuscript. Fernando D. Camargo is a Pew Scholar and is supported by grants from the National Institutes of Health, the Stand Up to Cancer Foundation and the Department of Defense.

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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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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_14,

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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Chapter 15
Hippo Signaling in Heart Development

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Abstract Although much has been learned about heart development, it is surprising that very little is known about how heart size is regulated. Recent work in mice revealed that Hippo signaling interacts with canonical Wnt/β-catenin signaling to regulate cardiac organ size during fetal development. Previous studies had shown that canonical Wnt/β signaling enhanced heart muscle or cardiomyocyte proliferation. As cardiomyocyte development progresses, Hippo signaling inhibits canonical Wnt/β-catenin activity through a multitiered mechanism to suppress cardiomyocyte proliferation and ultimately restrict heart size. These studies further demonstrate that Hippo and Warts kinases phosphorylate YAP to silence cardiomyocyte

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_15,

proliferation programs. Given the role of the Hippo pathway in cardiac size control, an important challenge for the future is to determine whether Hippo signaling also regulates cardiomyocyte proliferation in the normal and injured postnatal heart. Such studies may lay the groundwork for novel cardiovascular repair therapies. In this review, we will summarize the major developmental events that give rise to the vertebrate heart and highlight the established and potential roles that Hippo signaling serves during cardiogenesis to maintain proper organ size.

Keywords Heart size • Cardiomyocyte • Wnt signaling

15.1 Heart Development Overview

15.1.1 Current Understanding of Heart Muscle Development

In this short review, we will introduce heart development and focus on the developmental events and signaling pathways that are potentially important in heart size control and also have potential to directly interact with Hippo signaling in the heart. Although it is known that Hippo signaling is important in heart size regulation, an in-depth knowledge of Hippo in heart development and regeneration is lacking. Hippo signaling may potentially modulate any developmental mechanism that contributes to heart size control.

The heart develops from multiple mesodermal derivatives that give rise to the main cell types within the heart: myocardium, smooth muscle, fibroblasts, and endocardium. Among these cell types, the myocardium provides the main pumping action of the heart and is therefore the focus of intense research efforts. As might be expected, there are multiple signaling pathways that are important in regulating heart development.

The primary heart tube forms early in mouse embryogenesis from bilateral fields within the lateral plate mesoderm in what is known as the primary heart field.

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Multiple signaling interactions involving Bmp and Wnt signaling serve to both induce cardiogenic mesoderm and limit the cardiogenic induction to the anterior mesoderm (Foley and Mercola 2004). As the embryo turns and the pharynx develops further, the heart fields are pushed together and fuse to form a midline heart tube. At this stage, the heart begins a rightward looping motion that is an early manifestation of left–right asymmetry in the heart. The direction of heart looping is a late readout of multiple signaling events that initiate early in the mouse node, well before organogenesis, at pre-somite stages. The *Nodal-Pitx2* genetic pathway is a central component of this early signaling that establishes left–right identity within the heart. Nodal is a member of the Tgfβ superfamily of signaling factors that are important in many developmental contexts. Pitx2, a homeodomain transcription factor, is a direct transcriptional target for Nodal and a central effector of the left–right asymmetry pathways (Hamada et al. 2002).

Once the early heart tube has looped to the right, a second group of cardiac progenitors, referred to as the second heart field (SHF) moves out of the mesoderm posterior to the heart and into the outflow tract (OFT) and right ventricle. Some SHF-derived cells also contribute to the paired atria (Buckingham et al. 2005). Three groups first described the SHF independently in the chick and mouse model systems. This critically important discovery, uncovering for the first time that two heart fields contribute to myocardium, revealed that the heart is a modular structure comprised of cells with distinct developmental histories. Although present in primitive vertebrates, an expanded SHF is likely an evolutionarily recent event that enhanced the development of more complex cardiac morphologies seen in mammals.

15.2 Hippo Signaling in Heart Development

Although advances have been made, the mechanisms regulating heart muscle, or cardiomyocyte, proliferation remain poorly understood. The importance and interest in this problem is driven in part by the clinical need to develop strategies to replace injured myocardium after heart damage such as myocardial infarction. Recent work has uncovered a central role for Hippo signaling in regulating cardiomyocyte proliferation during heart development as a mechanism to control heart size (Heallen et al. 2011; Xin et al. 2011; von Gise et al. 2012).

Initial work looking at Hippo pathway components in the mammalian heart uncovered a role for the Hippo and Lats kinases in cardiac hypertrophy. Cardiac hypertrophy is a pathologic response to stress in which cardiomyocytes increase in cell size. Although initially physiologically beneficial, hypertrophy eventually leads to heart failure and is a leading cause of morbidity and mortality in the human population. Gain of function and dominant negative approaches revealed that Hippo and Lats kinases regulated cardiac hypertrophy under stressed conditions (Matsui et al. 2008; Yamamoto et al. 2003). These intriguing, groundbreaking experiments set the stage for loss of function genetic experiments investigating Hippo signaling in cardiac development.

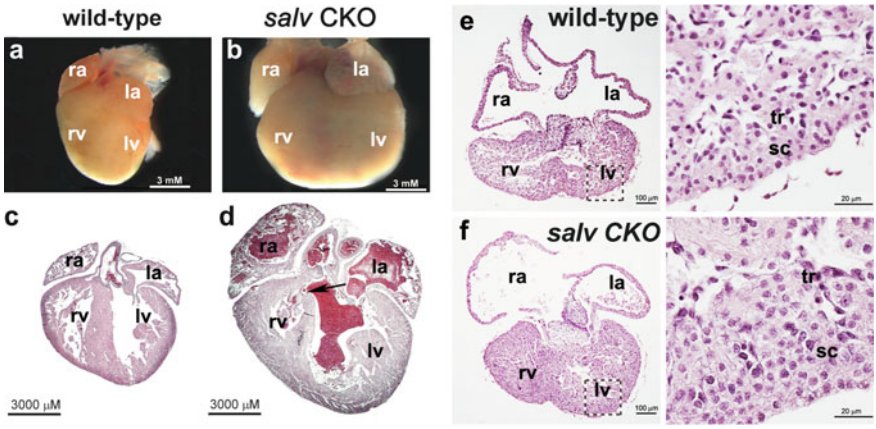

Fig. 15.1 Salvador mutant cardiomegaly. (**a**–**d**) Control ((**a**) and (**c**)) and *Salv CKO* ((**b**) and (**d**)) P2 neonate hearts. ra indicates right atrium; la, left atrium; rv, right ventricle; lv, left ventricle. Hearts in (**a**) and (**b**) were sectioned and stained with hematoxylin and eosin, as shown in (**c**) and (**d**). *Arrow*, ventricular septum defect. (**e** and **f**) hematoxylin and eosin stained control (**e**) and *Salv CKO* (**f**) hearts. High magnification of (**e**) and (**f**) are shown in the right-hand images; subcompact, sc; trabecular, tr myocardium. Control genotype is *Nkx2.5*^{cre}; *Salv*$^{fl+}$

Heallen et al. (2011) used gene inactivation studies in mice to delete Hippo pathway kinases and adaptor molecules in the heart. These studies revealed Hippo signaling as a critical developmental pathway that restricts cardiomyocyte proliferation (Heallen et al. 2011). As cardiac chamber formation commences, Hippo pathway activity functions as a restraint mechanism for cardiomyocyte proliferation in the ventricular myocardium. Mice that are cardiac-deficient for the Mst adaptor Salvador (*Salv CKO*) develop severe cardiomegaly, exhibiting a near 2.5-fold increase in heart size at birth (Fig. 15.1). Although having hypercellular, thickened ventricular walls by embryonic day 12.5 (E12.5), chamber formation and tissue patterning remain grossly unaffected. Investigation into the *Salv CKO* hyperplasia phenotype revealed that ventricular septation defects arise, albeit at a very low frequency (Fig. 15.1). Cardiomyocyte proliferation was elevated fourfold in the *Salv CKO* mutant cardiomyocytes but proliferation of other cardiac cell types was unaffected. Moreover, cardiomyocyte cell size in *Salv CKO* hearts was also unchanged from control. Deletion of *Mst1/2* or *Lats 2* in embryonic hearts yielded similar, although more severe phenotypes as the *Salv* deletion. Deletion of Salvador severely diminishes phosphorylated YAP levels in these hearts, indicating a conserved Hippo-YAP regulatory cassette in the developing heart.

Expression profiling of E9.5-stage Hippo-deficient hearts revealed that transcript levels of genes involved in promoting cell cycle progression such as *Cyclin D1* were elevated. Interestingly, genes involved in cellular reprograming, such as *Sox2*, and epithelial to mesenchymal transition, such as *Snai2*, were also upregulated in the *Salv CKO* hearts (Heallen et al. 2011; Koyanagi et al. 2010; Takahashi and Yamanaka 2006; Cano et al. 2000). Apoptosis regulators, such as the apoptosis inhibitor Birc5

or Survivin, were also elevated in the *Salv CKO* heart. In addition, a large subset of canonical Wnt/β-catenin regulated genes are expressed at high levels in *Salv CKO* hearts (see below).

Consistently, other reports reveal that the Hippo effector YAP is necessary and sufficient for cardiomyocyte proliferation in the fetal heart (von Gise et al. 2012; Xin et al. 2011). These studies revealed that embryonic deletion of YAP leads to mid- to late-stage lethality marked by ventricular myocardial hypoplasia and contractile dysfunction due to reduced cardiomyocyte proliferation (von Gise et al. 2012; Xin et al. 2011). By contrast, cardiac-specific transgenic-overexpression of YAP produced large hearts having enhanced cardiomyocyte proliferation (von Gise et al. 2012; Xin et al. 2011). These strains (YapS112A Tg and YapS127A Tg respectively) express a constitutively active form of Yap in which a major Serine residue for Lats phosphorylation is mutated to Alanine and thereby permits Yap to accumulate in the nucleus (Camargo et al. 2007; Nishioka et al. 2009). The cardiac phenotypes of YapS112A Tg and YapS127A Tg mice are strikingly reminiscent of *Salv CKO* hearts in that cardiomyocyte proliferation is upregulated in the myocardium and chamber walls are thickened and hypercellular, most significantly in the trabecular layer (von Gise et al. 2012; Xin et al. 2011). Hence, YAP is a critical factor that regulates cardiomyocyte proliferation during development.

Viral infection of an activated form of human YAP1 (S127A) into both fetal and postnatal rat cardiomyocytes revealed that Yap1 is sufficient to trigger cardiomyocyte proliferation. In addition, expression levels of mitotic and cytokinesis genes are elevated in YapS112A Tg and YapS127A Tg neonatal hearts (von Gise et al. 2012; Xin et al. 2011). Microarray profiling revealed increased expression of cell cycle genes *Cyclin A2*, *Cyclin B1*, and *Cyclin-dependent kinase 1* in Yap-overexpressing cardiomyocytes concomitant with increased cell-cycling (von Gise et al. 2012; Xin et al. 2011). These findings suggest an underlying postnatal mechanism whereby YAP promotes cardiomyocyte proliferation via direct activation of growth factors and cell cycle progression of cardiomyocytes. In cardiomyocytes where YAP activity is high, apoptosis levels are reduced (von Gise et al. 2012; Xin et al. 2011). This is consistent with the observation that expression levels of anti-apoptosis factors, such as Birc5, are upregulated in *Salv CKO* hearts (Heallen et al. 2011). Interestingly, the size of YapS112A Tg hearts normalizes to wild-type dimensions by adulthood despite having an increased number of cardiomyocytes in a given cross-sectional area (Xin et al. 2011). The authors speculate that a growth compensatory mechanism likely exists and is activated in these hearts to maintain proper heart size.

Tead/Tef transcription factors appear to serve as major binding partners of Yap during cardiomyocyte growth (Heallen et al. 2011; von Gise et al. 2012). Yap-induced expression of cell cycle genes, in addition to cardiomyocyte proliferation, requires the YAP-Tead1 interaction (von Gise et al. 2012). Moreover, full activation of *Sox2* and *Snai2* transcriptional reporters is dependent on Tead2 (Heallen et al. 2011). Given these data, it appears that during cardiac development Tead factors are the primary DNA-binding cofactors that function with Yap to regulate cardiac proliferation and organ size.

15.3 Hippo Restricts Canonical Wnt Signaling

Throughout mammalian life, canonical Wnt/β-catenin pathway signaling has key roles in a wide spectrum of biological processes (Clevers and Nusse 2012). Deregulation of Wnt/β-catenin signaling is observed in a number of pathologies including cancer, osteoporosis, and a host of congenital and developmental disorders (Clevers and Nusse 2012). Wnt ligand binding of Frizzled receptors triggers phosphorylation of the cytoplasmic signal transducer Dishevelled (DVL) (McNeill and Woodgett 2010). In a poorly understood mechanism, phosphorylated DVL interacts with and inhibits the GSK-3β-Axin-APC degradation complex (McNeill and Woodgett 2010). In the absence of Wnt signaling, this complex functions to keep cytosolic and nuclear β-catenin levels low by promoting its phosphorylation and ubiquitination (Nusse 2005). When the pathway is active, β-catenin is stabilized and associates with Tcf/Lef DNA-binding factors in the nucleus to activate transcription of growth-genes (Nusse 2005).

In the heart, Wnt signaling has several highlighted roles including progenitor cell renewal and differentiation and cardiac morphogenesis (Cohen et al. 2008). Wnt signaling also has context-dependent roles in myocardial development. In early heart development during primary heart field specification, Wnt signaling represses cardiac mesoderm development. In contrast to its negative role in primary heart field, in SHF, Wnt signaling enhances SHF progenitor expansion. As cardiomyocyte progenitors leave the SHF and enter the heart proper, Wnt signaling activates genes such as *Cyclin D2*, *Isl1*, and *Fgf10* to enhance cell cycle progression and stabilization of the SHF cell state.

The crosstalk between the Wnt/β-catenin and Hippo pathways is highly complex. The current data support a model whereby Hippo signaling inhibits Wnt activity via a multitiered mechanism (Fig. 15.2). At the intersection of these pathways are the YAP/TAZ and β-catenin effector molecules.

Experiments performed in the context of kidney development indicate that TAZ when phosphorylated by Lats2 can directly interact with DVL in the cytoplasm to inhibit activation of Wnt signaling (Varelas et al. 2010a). In TAZ mutant kidneys, β-catenin is redistributed from cell membrane junctions and becomes increasingly cytoplasmic and nuclear (Varelas et al. 2010a). In addition, siRNA-knockdown of TAZ induces Wnt reporter activity and target gene expression (Varelas et al. 2010a). Comprehensive biochemical analyses in this context revealed that phosphorylated TAZ binds DVL in the cytoplasm thereby preventing CK1δ/ε binding and Wnt-dependent phosphorylation of DVL. Moreover, Hippo signaling induces TAZ phosphorylation and cytoplasmic localization, leading to the inhibitory TAZ-DVL interaction. Altogether, these findings identify a Hippo-TAZ-DVL negative interaction in the cytoplasm to inhibit the cellular response to Wnt signaling.

Other recent data, obtained in cultured cells and Xenopus embryos, provide a conceptually similar mechanism whereby phospho-Yap interacts with β-catenin in the cytoplasm to prevent nuclear accumulation of β-catenin and thereby dampen the cellular response to Wnt/β-catenin signaling. Hippo signaling induces phosphorylation

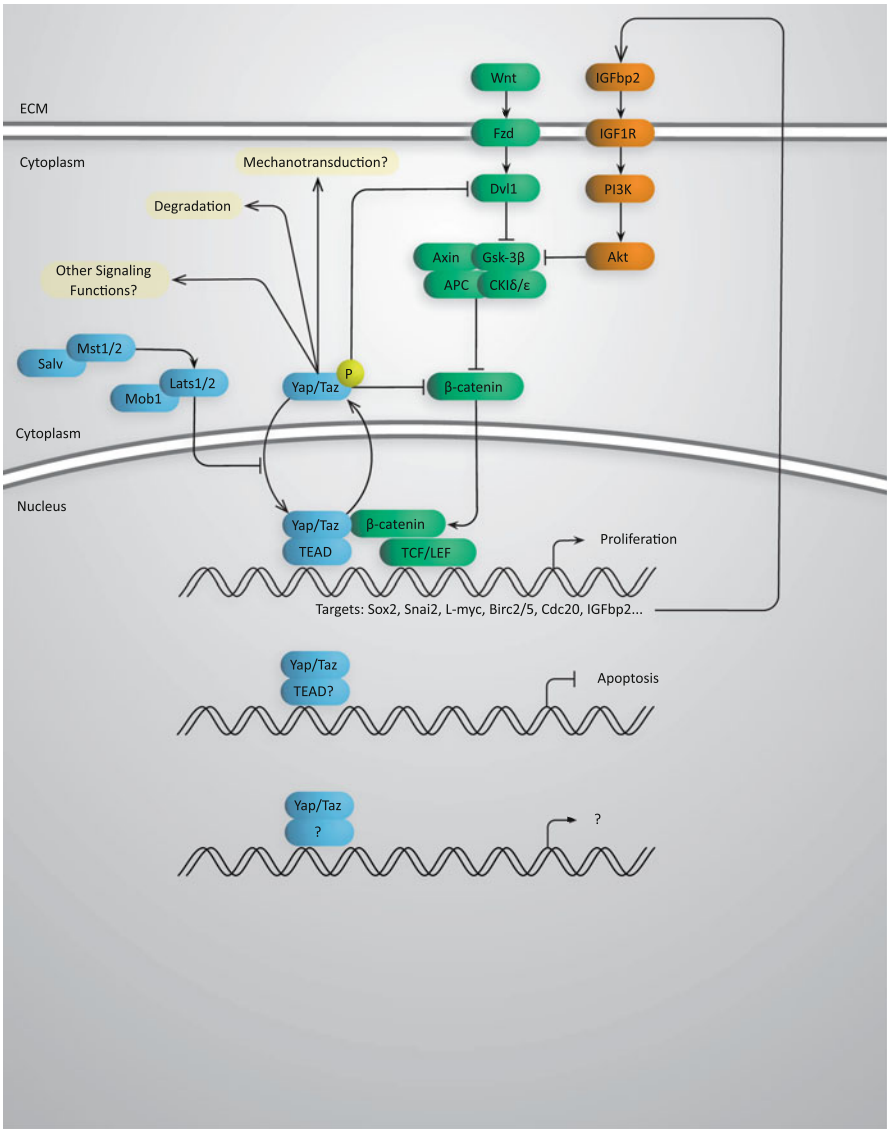

Fig. 15.2 Convergence of Hippo and Wnt signaling pathways via Yap/Taz. Integration of the Hippo Pathway (Mst1/2 & Lats1/2) with the Wnt (Wnt, Fzd, Dvl, & Gsk-3β) and Insulin-like growth factor (IGF) pathways (IGF, IGFR, PI3K, & Akt) is known to effect cardiomyocyte proliferation. The Hippo pathway regulation of Yap target gene expression in the heart is mediated through Yap/Taz/TEAD and β-catenin/TCF target gene expression. Yap/Taz and TEAD elements work in conjunction with β-catenin and TCF/LEF to initiate transcriptional programs leading to increased proliferation. It is known that this combination leads to increases in IGFbp2. Through a positive feedback IGF signaling leads to Akt-mediated inactivation of Gsk-3β, having the effect of increased β-catenin. Active Hippo signaling leads to phosphorylation of Yap/Taz and subsequent retention in the cytoplasm. Phosphorylated Yap/Taz act directly and indirectly to lower nuclear β-catenin levels. Directly by binding to and sequestering β-catenin in the cytoplasm, and indirectly through inhibition of Dvl1 activity upstream. Emerging evidence suggests a function of Yap at the plasma membrane in mechano-transduction

and cytoplasmic localization of YAP/TAZ where these molecules bind to β-catenin and prevent nuclear accumulation (Imajo et al. 2012). The Tead-binding domain of YAP is critical for binding the N-terminal region β-catenin and suppression of Wnt/β-catenin target gene expression (Imajo et al. 2012). In this context, Hippo signaling through phospho-Yap prevents nuclear translocation of β-catenin, rather than affecting β-catenin protein stability (Imajo et al. 2012). Remarkably, these findings also revealed that phospho-Yap prevented nuclear accumulation of a constitutively active form of β-catenin suggesting that Hippo signaling is dominant to Wnt signaling in β-catenin regulation.

In the heart, several lines of evidence indicate that Hippo signaling inhibits Wnt/β-catenin-dependent cardiac muscle growth. *Salv CKO* hearts exhibit upregulated expression of β-catenin target genes *Sox2*, *Snai2*, and *Birc5* (Heallen et al. 2011). Similarly, *Salv CKO* myocardium displays a fourfold increase in the nuclear β-catenin index (Heallen et al. 2011). Importantly, genetic interaction studies indicate that the Hippo cardiomyocyte overgrowth phenotype is suppressed by β-catenin haploinsufficiency. Furthermore, biochemical assays revealed that Yap complexes with β-catenin on promoter elements of *Sox2* and *Snai2* genes (Heallen et al. 2011). In the context of heart development, Hippo signaling prevents the interaction of Yap with β-catenin in the nucleus to promote transcription of growth-genes. Hence, Hippo prevention of Yap nuclear function is a critical junction for mediating Hippo-Wnt crosstalk in the heart (Fig. 15.2). Other work suggests that in cell types other than the heart, the Yap-β-catenin coordinate regulation of target genes may be a less important mechanism for Wnt-Hippo crosstalk (Imajo et al. 2012).

Gene expression studies in the YapS112A Tg fetal heart revealed that YAP induces expression of genes in the Insulin-like growth factor (IGF) signaling pathway, a regulatory network having established regulatory roles in during cardiac growth (Xin et al. 2011). Genes induced by Yap include *IGF1*, *Igfbp2*, *Igfbp3* in addition to β-catenin and its downstream target genes. Functional studies reveal that IGF pathway activity is stimulated in YapS112A-expressing cardiomyocytes, evidenced by increased levels of P13K and phosphorylated Akt (Xin et al. 2011). IGF signaling induces Akt-mediated phosphorylation of the β-catenin destruction complex component GSK-3β resulting in GSK-3β inactivation and β-catenin stabilization (Xin et al. 2011). Indeed, in YapS112A-expressing cells, levels of phosphorylated Akt and GSK-3β are enhanced in addition to accumulation of non-phosphorylated and active β-catenin (Xin et al. 2011). These findings collectively define an additional mode of Hippo-mediated Wnt inhibition via YAP activation of the IGF pathway (Fig. 15.2).

Another recently reported mechanism indicates that in colorectal carcinoma cells, β-catenin/TCF4 binds a DNA enhancer element residing in YAP's first intron to promote YAP expression (Konsavage et al. 2012). This finding suggests a YAP-β-catenin positive feedback mechanism when Hippo activity is low.

As summarized in Fig. 15.2, Yap/Taz integrates the Hippo, Wnt, and IGF signaling pathways. When active as a transcription factor, Yap/Taz interacts with β-catenin to control TEAD/TCF common target genes. In Hippo-deficient cardiomyocytes,

Yap/Taz works in conjunction with TEAD elements, β-catenin, and TCF/LEF to activate genes that promote proliferation resulting in increased heart size. YAP promotes IGF1 pathway signaling which feeds back to the Wnt pathway via Akt-mediated inactivation of Gsk-3β, leading to stabilized β-catenin. Hippo signaling-mediated Yap/Taz phosphorylation (pYAP/pTAZ) resulting in pYAP/pTAZ cytoplasmic sequestration prevents cooperative interactions with nuclear β-catenin. Experiments performed in noncardiac cells suggest that in the heart pYap/pTaz may bind cytoplasmic β-catenin, preventing β-catenin nuclear localization to further reduce β-catenin transcriptional activity. Additionally, pYap/pTaz inhibits Dvl1 in a separate mechanism to control β-catenin. Thus, YAP and TAZ serve to integrate Hippo signaling via multiple mechanisms to regulate β-catenin activity.

15.3.1 Other Signaling Pathways in Myocardial Development

Bone morphogenetic protein (Bmp) signaling has multiple, stage-specific roles in heart development. The Bmp receptors, serine-threonine kinases, activate a signal transduction pathway that utilizes Smad effector molecules to regulate gene transcription. In the Bmp pathway, the receptor regulated Smads, Smad1, Smad5, and Smad8 are phosphorylated and interact with the common Smad4. The Smad complexes undergo nucleo-cytoplasmic shuttling with nuclear localization promoted by Bmp signaling. Importantly, Smad effectors have been shown to directly interact with Yap and Taz uncovering a direct interaction between Hippo and Bmp signaling in certain contexts (Alarcon et al. 2009; Varelas et al. 2008, 2010b).

At early stages, Bmp signaling plays a role in cardiogenic mesoderm induction. Using the chick system, it was shown that Bmp2 expressed in endoderm signals to anterior lateral plate mesoderm to induce the *Nkx2.5*-expressing cardiogenic mesoderm (Schultheiss et al. 1997). As cardiac progenitors transition to the more differentiated but still immature myocardium, Bmp signaling is also essential to promote myocardial differentiation. An exquisite series of experiments uncovered a *Bmp2-Nkx2.5* feedback loop that is a critical component of the progenitor to myocardial transition (Prall et al. 2007). In this model, *Nkx2.5* suppresses *Bmp2* that promotes myocardial specification and concurrently shuts down cardiac progenitor proliferation. The *Nkx2.5-Bmp2* negative feedback loop provides a mechanism that coordinates cardiomyocyte specification, the size of the cardiomyocyte progenitor pool, and right ventricular size. Importantly, this work also suggests that organ size control mechanisms are present in E8.5-9.5 SHF cardiac progenitors raising the possibility that Hippo signaling and Yap may have a role in heart size control at these early stages.

Continuing the theme of negative feedback regulatory mechanisms in cardiac progenitors, a second study uncovered a Bmp-microRNA (miR) pathway that inhibits progenitor genes like *Isl1* and *Tbx1* to promote the transition from progenitor cell to differentiated myocardium. In this model, Bmp signaling directly regulates

miR-17-92 complex transcription in SHF progenitors and also primitive cardiomyocytes (Wang et al. 2010). The *miR-17-92* complex encodes six mature miRs that repress progenitor gene expression in the SHF and OFT myocardium (Ventura et al. 2008). Genes such as *Isl1* and *Tbx1*, encoding transcriptional regulators that are critical for normal SHF development, are direct targets for *miR-17-92*. Bmp signaling is also important at later stages in cardiac development, after the myocardium is established. Bmp10 signaling is important in enhancing proliferation of chamber myocardium (Chen et al. 2004). It will be important in future studies to determine whether the Hippo-Yap pathway intersects with these characterized Bmp-mediated mechanisms that control heart size.

Notably, evaluation of Hippo activity using a Phospho-Yap antibody suggested that Hippo signaling was active at E9.5 in SHF progenitors. Despite this, no SHF phenotypes were observed in the Hippo-deficient embryos when examined at E9.5 (Heallen et al. 2011). Together these findings suggest that there may be redundancy in Hippo pathway kinases during early heart development that obscures phenotypic analyses. Alternatively, Hippo-independent mechanisms may exist in the SHF to exclude Yap from the nucleus. Consistent with this reasoning, it has been shown in epithelial contexts that Yap is excluded from the nucleus through interaction with catenins in the adherens junction (Schlegelmilch et al. 2011). Other similar mechanisms for sequestering Yap from the nucleus have been observed and recently reviewed (Boggiano and Fehon 2012). Future experiments will be directed at investigating the potential for Hippo-independent regulation of Yap in the SHF. Also because Hippo signaling is a critical regulator of heart size, an important area for future investigation will be to investigate the connection between Hippo signaling and the *Nkx2.5-Bmp2* feedback loop that is involved in regulation of right ventricle size.

15.4 Final Considerations and Future Directions

Recent years have seen an explosion of research into the Hippo signaling pathway. It is now clear that these rapid advances will also lead to important new advances in cardiomyocyte biology. A top priority will be to study Hippo signaling in mammalian adult cardiomyocytes. Unlike the amphibian or fish heart, mammalian hearts have limited regenerative capacity (Bergmann et al. 2009). Cardiac regeneration has been extensively studied in zebrafish where myocardium regenerates after resecting 20 % of the ventricles (Choi and Poss 2012). During this process, the amputated region of the heart is repopulated with cardiomyocytes that are derived from preexisting cardiomyocytes through dedifferentiation. Recently, it was shown that the rodent heart has regenerative ability until postnatal day 7 through a similar mechanism to fish heart regeneration (Porrello et al. 2011). It will be important to determine whether Hippo pathway inactivation in postnatal and adult mammalian cardiomyocytes can extend the period of mammalian cardiac regeneration.

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Part VI
Cross-Talk with Other Pathways

Chapter 16
Cell Competition and the Hippo Pathway

Chiao-Lin Chen and Georg Halder

Abstract Cell competition was discovered in *Drosophila* as a phenomenon whereby slow growing cells are eliminated from developing tissues. Competitive cell–cell interactions can also eliminate cells with other defects such as defects in cell polarity. Cell competition is thus regarded as a homeostatic mechanism that eliminates abnormal and potentially harmful cells. Notably, the elimination of abnormal cells by cell competition is only observed in mixed cell populations and may thus depend on specific and reciprocal cell–cell interactions. However, many questions about the mechanisms of cell competition remain. For example, what determines the competitive status of a cell and how do cells detect differences in fitness? Recent work has identified the Hippo tumor suppressor pathway as a regulator and effector of cell competition in *Drosophila*. In this chapter, we discuss how the Hippo pathway is linked to cell competition and how this connection may act as a tumor suppressor mechanism.

16.1 Classic Cell Competition: Minute and Myc

Cell competition was first observed in *Drosophila*: When mosaic flies were generated that contained two genotypes of cells with different growth ability, the slower growing cell population was progressively eliminated during development and the adult tissues were composed predominantly of the faster growing cells (Morata and Ripoll 1975). This may not be trivial because the slow growing cells were able to

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_16,

give rise to adults when the animals were comprised exclusively of the slow growing cells, although it took these animals longer to reach adulthood. From these results it follows that the behavior of a cell depends on its context: a cell may survive in a homotypic environment but die when juxtaposed to fitter cells. Thus, the elimination of the slow growing cell population depends on the presence of the faster growing cells, which "outcompete" the slow growing cells, hence the name "cell competition."

During cell competition, the less competitive cells are typically eliminated by apoptosis while the more competitive cells can engulf the dying cells and undergo extra rounds of proliferation to make up for the loss of the weaker cells (reviewed in Baker 2011; Baker and Li 2008; de Beco et al. 2012; Moreno 2008; Tamori and Deng 2011; Vivarelli et al. 2012). Cells that survive and proliferate at the expense of other cells are called "winners," while cells that are eliminated are called "losers."

The classically used system to study cell competition are the imaginal discs of *Drosophila*, epithelial tissues that give rise to many of the external structures of the adult fly (reviewed in Neto-Silva et al. 2009). The imaginal discs proliferate during the larval stages and terminally differentiate during metamorphosis in the pupal stage. Cell competition is assayed in imaginal discs that are genetic mosaics, meaning that they are composed of two cell populations with different genotypes (reviewed in Blair 2003). Mosaic imaginal discs thus have patches or "clones" of mutant cells embedded in a background of phenotypically wild-type cells for example. Classic examples of mutations that affect cell competition are mutations in a class of genes known as *Minutes* (*M*) (Morata and Ripoll 1975). *M* are loss of function mutations in ribosomal genes that cause a dominant slow growth phenotype in heterozygous cells, presumably due to reduced translational efficiency (Lambertsson 1998; Marygold et al. 2007). Cells that are homozygous for an *M* mutation are not viable, but heterozygous $M^{+/-}$ cells can form adult tissues, although they take longer than normal (Morata and Ripoll 1975; Simpson 1979; Simpson and Morata 1981). Notably, however, $M^{+/-}$ cells survive only when all cells in the tissue are $M^{+/-}$, while they are eliminated when wild-type cells are present. The elimination of $M^{+/-}$ cells involves apoptosis, which is induced in $M^{+/-}$ cells mainly along the borders of clones of $M^{+/-}$ cells (Li and Baker 2007; Li et al. 2009; Martin et al. 2009; Moreno et al. 2002), and blocking apoptosis by overexpressing the cell death inhibitor p35 rescues $M^{+/-}$ cells from elimination (Li and Baker 2007; Li et al. 2009). Outcompeted cells are removed by engulfment which may be done by neighboring wild-type cells (Li and Baker 2007) or by hemocytes after apoptosis and extrusion from the epithelium (Lolo et al. 2012). On the other hand, neighboring non-competed cells undergo compensatory proliferation to replace the outcompeted cells (Simpson 1979; Simpson and Morata 1981). Thus, $M^{+/-}$ cells are eliminated when juxtaposed to wild-type cells, which proliferate at their expense. Consequently, the tissues of mosaic adults are composed of mainly wild-type cells with few $M^{+/-}$ cells present.

Similar to the effects of *M* mutations, hypomorphic loss of function of the *Drosophila* Myc homolog *dMyc* (also called *diminutive*) causes reduced cell growth and proliferation and reduced competitive fitness such that homozygous *dMyc*

mutant cells are outcompeted in mosaic tissues (Johnston et al. 1999). dMyc is a transcription factor that promotes cellular growth, proliferation, and ribosomal biogenesis by regulating the expression of a battery of genes including ribosomal genes (Grandori et al. 2005; Grewal et al. 2005; Orian et al. 2003, reviewed in de la Cova and Johnston 2006). The finding that dMyc regulates ribosomal biogenesis and that its effects on cell competition depend on normal levels of M expression (Moreno and Basler 2004) may explain the similarity of the cell competition phenotypes of *dMyc* and $M^{+/-}$ mutations, although dMyc may affect cell competition through other targets as well. In summary, wild-type cells are winners when they are mixed with $dMyc^{-}$ or $M^{+/-}$ mutant cells.

Conversely to its loss of function phenotypes, dMyc overexpression turns cells into super-competitors, which are more competitive than wild-type cells (de la Cova et al. 2004; Moreno and Basler 2004). Wild-type cells that are juxtaposed to cells with high levels of dMyc are thus outcompeted, becoming loser cells in this scenario. This example shows that "loser" and "winner" are not absolute properties of cells but depend on the context as wild-type cells are winners when mixed with cells that have reduced levels of dMyc but losers when mixed with cells that overexpress dMyc. It is therefore the relative fitness of different cell populations that is detected by cell competition and that ultimately dictates cell survival (Baker 2011; Baker and Li 2008; de Beco et al. 2012; Moreno 2008; Tamori and Deng 2011; Vivarelli et al. 2012).

The observation that $M^{+/-}$ cells are slow growing and that dMyc promotes cell growth and proliferation suggests that cellular fitness may simply be related to the growth rate of a cell. However, alternate approaches to increase cell growth rates such as hyperactivation of the PI3Kinase pathway by overexpression of Dp110, the catalytic subunit of PI3Kinase, or overexpression of the growth regulator Cyclin D with its kinase partner Cdk4, does not turn cells into super-competitors even though such mutant cells are larger and proliferate faster than wild-type cells (de la Cova et al. 2004). Thus, the fitness of a cell is not simply proportional to its growth rate and additional factors must exist that determine cellular fitness.

Cell competition is not only observed in *Drosophila* imaginal discs but also in other tissues such as in the *Drosophila* germ line stem cell (GSC) niche and in the follicular cell epithelium which surrounds growing oocytes where dMyc induces cell competition (Froldi et al. 2010; Rhiner et al. 2009), in rodents (Bondar and Medzhitov 2010; Oertel et al. 2006; Oliver et al. 2004) and in human tissue culture cells (Bondar and Medzhitov 2010; Norman et al. 2012; Tamori et al. 2010). In the *Drosophila* ovary, GSCs compete with each other such that GSCs with relatively lower levels of dMyc expression are replaced by GSCs with higher levels of dMyc expression (Rhiner et al. 2009). Like in imaginal discs, overexpression of dMyc is sufficient to turn GSCs into super-competitors that outcompete wild-type GSCs, and GSCs hypomorphic for *dMyc* are lost over time. Similar to the situation in imaginal discs, hyperactivation of the PI3Kinase pathway by loss of Pten did not affect the competitive capacity of a GSC even though *Pten* mutant GSCs proliferated faster and grew larger than wild-type GSCs. In contrast to imaginal discs, however, loser GSCs were not eliminated by apoptosis but presumably expelled from the niche as no evidence for apoptosis was found and expression of the apoptosis inhibitor

Drosophila Inhibitor of Apoptosis Protein-1(DIAP1) did not prevent the loss of loser cells (Rhiner et al. 2009). Thus, cell competition may not necessarily require elimination by apoptosis.

In addition to the classic *M* and *dMyc* cases, cell competition is triggered by defects in signaling pathways that are important for tissue patterning and growth such as the Wnt, Dpp, JAK/STAT, and Hippo pathways or when cell polarity is lost (Brumby and Richardson 2003; Moreno et al. 2002; Neto-Silva et al. 2010; Rodrigues et al. 2012; Tyler et al. 2007; Vincent et al. 2011; Ziosi et al. 2010). Notably, imaginal disc cells with hyperactivated Wnt or JAK/STAT signaling behave as super-competitors, but independently of dMyc, illustrating that cell competition may not encompass a single molecular mechanism but may involve multiple pathways (Rodrigues et al. 2012; Vincent et al. 2011). The discovery of phenomenologically similar processes that eliminate cells with other defects has thus led to an expansion of the term cell competition (Baker 2011; Baker and Li 2008; de Beco et al. 2012; Moreno 2008; Tamori and Deng 2011; Vivarelli et al. 2012). The term cell competition is nowadays used more broadly and refers to context-dependent interactions between two cell populations that cause a change in survival or growth rate of one or both cell populations. In the next sections we discuss recent advances in our understating of cell competition with special emphasis on the involvement of the Hippo pathway.

16.2 The Hippo Pathway Regulates Cell Competition

The Hippo signal transduction pathway was identified due to its dramatic growth phenotypes in *Drosophila* where mutations that inactivate the pathway cause severe overgrowth of imaginal discs and corresponding adult structures (reviewed in Enomoto and Igaki 2011; Genevet and Tapon 2011; Grusche et al. 2010; Halder and Johnson 2011; Pan 2010; Staley and Irvine 2012; Zhao et al. 2011). These overgrowth phenotypes of *hpo* mutant imaginal discs are caused because mutant cells undergo excessive proliferation and are resistant to signals that normally eliminate excess cells in imaginal discs. Notably, depleting Hippo pathway activity in imaginal discs leads to massive overgrowth, but with little patterning defects or cell size changes. Hippo signaling thus coordinately regulates cell proliferation and apoptosis, processes that are critical for the proper determination of organ size. The Hippo pathway is conserved in vertebrates where it also regulates cell proliferation and organ size: For example, conditional ablation of the Hpo homologs *mst1* and *mst2* or overexpression of the Hippo pathway effector Yap during mouse liver development causes enlarged livers (Dong et al. 2007; Lu et al. 2010; Song et al. 2010). Importantly, defects in Hippo signaling are associated with the development of several different types of cancer (reviewed in Chen et al. 2012b; Pan 2010; Zhao et al. 2011). The Hippo pathway is thus a conserved signal transduction pathway that regulates tissue growth and is an essential tumor suppressor pathway. The Hippo pathway, however, does not only regulate cell proliferation and organ size but it is also involved in several other processes including cell competition (Neto-Silva et al.

2010; Tyler et al. 2007; Ziosi et al. 2010). Interestingly, the Hippo pathway may not only be an upstream regulator of cell competition, but its activity may itself be a target of cell competition (Chen et al. 2012a).

Molecularly, the Hippo pathway has emerged as a network of components that transduce extracellular signals and mechanical cues from the plasma membrane into the nucleus where it regulates gene expression (reviewed in Enomoto and Igaki 2011; Genevet and Tapon 2011; Grusche et al. 2010; Halder et al. 2012; Halder and Johnson 2011; Pan 2010; Schroeder and Halder 2012; Staley and Irvine 2012; Zhao et al. 2011). The core of the pathway forms a kinase cascade of the Hippo (Hpo; MST1 and MST2 in vertebrates) and Warts (Wts; LATS1 and LATS2 in vertebrates) serine/threonine kinases and their cofactors Salvador (Sav; SAV1 in vertebrates), and Mob as a tumor suppressor (Mats; MOB1 in vertebrates), respectively. Wts phosphorylates and inhibits the activity of the transcriptional co-activator Yorkie (Yki; YAP and TAZ in vertebrates) by regulating its nuclear localization. Phosphorylated Yki/YAP/TAZ remain in the cytoplasm whereas unphosphorylated Yki/YAP/TAZ translocate into the nucleus and form complexes with different transcription factors such as Scalloped (Sd, TEAD1-4 in vertebrates) to induce the expression of target genes.

The effect of the Hippo pathway on cell competition has been studied in the context of mosaic *Drosophila* imaginal discs. Cells that have hyperactive Yki due to mutation in a Hippo pathway component not only overproliferate but they are super-competitors and outcompete wild-type cells (Neto-Silva et al. 2010; Tyler et al. 2007; Ziosi et al. 2010). Clones of cells mutant for the Hippo pathway induced elevated levels of apoptosis in neighboring wild-type cells and reduced their proliferation (Neto-Silva et al. 2010; Tyler et al. 2007; Ziosi et al. 2010). In addition to this direct demonstration of the effects of Hippo signaling on cell competition, the Hippo pathway has been linked to cell competition because loss of Hippo signaling can rescue $M^{+/-}$ cells from being outcompeted (Tyler et al. 2007). A genetic screen for mutations that can rescue $M^{+/-}$ cells from cell competition identified mutations in several components of the Hippo pathway including *fat*, *ex*, *sav*, *hpo*, and *wts* (Tyler et al. 2007). Thus $M^{+/-}$ cells that are at the same time double mutant for Hippo signaling are not eliminated in genetic mosaics. In contrast, manipulation of other growth control or patterning pathways, including for example activation of the PI3Kinase, TOR, and Ras/MAPKinase pathways, did not affect the competition between wild-type and $M^{+/-}$ cells (Tyler et al. 2007). This specificity indicates that the effect of Hippo signaling on cell competition may be specific rather than a secondary side effect caused by the elevated rate of cell proliferation in *hpo* mutant cells.

16.3 The Hippo Pathway Affects Cell Competition Through Regulating Myc Expression

A breakthrough in understanding how the Hippo pathway regulates cell competition came with the discovery that Yki directly regulates the expression of *dMyc* (Neto-Silva et al. 2010; Ziosi et al. 2010). Imaginal discs with clones of

cells that were mutant for *hpo* or *wts*, or that overexpressed Yki had elevated levels of dMyc protein as well as mRNA, while cells with hyperactivated Hippo signaling due to overexpression of the Hpo kinase, or cells with knocked-down amounts of Sd, had lower levels of dMyc. Further, Yki/Sd complexes bound to an enhancer element in the *dMyc* gene that was activated by Yki/Sd in a tissue culture reporter assay (Neto-Silva et al. 2010; Ziosi et al. 2010). Yki/Sd thus directly regulate the expression of *dMyc*.

The Hippo pathway is known to regulate the expression of a battery of downstream target genes, many of which promote cell proliferation and cell survival, including the cell cycle gene *CyclinE* (*CycE*), the anti-apoptotic gene *diap1*, the growth promoting and anti-apoptotic microRNA *bantam*, and *dMyc* (Harvey et al. 2003; Neto-Silva et al. 2010; Nolo et al. 2006; Thompson and Cohen 2006; Udan et al. 2003; Wu et al. 2003; Ziosi et al. 2010). dMyc is required for the growth and proliferation of normal cells and similarly, dMyc is essential for the growth of cells with hyperactivated Yki (Neto-Silva et al. 2010; Ziosi et al. 2010). Thus, Yki overexpressing cells that are mutant for *dMyc* are unable to grow and form only very small clones similar to the phenotype of *dMyc* mutant cells. Therefore, dMyc is required for Yki to drive tissue growth indicating that dMyc is an important downstream target gene of the Hippo pathway. On the other hand experimental overexpression of dMyc in *yki* mutant cells was not sufficient to rescue the growth defects of *yki* mutant cells, indicating that the regulation of other targets is also essential for Yki's growth promoting function.

The regulation of multiple target genes may contribute to the growth promoting effects of Yki (Neto-Silva et al. 2010; Nolo et al. 2006), however, the effects of the Hippo pathway on cell competition may largely, if not entirely, depend on its regulation of dMyc expression (Ziosi et al. 2010). The super-competitive status of Yki overexpressing cells was diminished when they were confronted with cells that had experimentally elevated levels of dMyc expression, such that the difference in dMyc levels between Yki expressing cells and surrounding cells was minimized (Ziosi et al. 2010). Moreover, reducing the levels of dMyc in Yki expressing cells by halving the gene dose of dMyc further diminished the competitive ability of Yki expressing cells. These results indicate that the elevated dMyc expression in Yki expressing cells compared to their surrounding cells is important and possibly sufficient for their super-competitive status (Ziosi et al. 2010).

Interestingly, dMyc regulates the levels of Yki in a negative feedback loop (Neto-Silva et al. 2010). Overexpression of dMyc in wing imaginal discs caused reduced Yki protein levels and conversely, knockdown of dMyc resulted in cells with higher levels of Yki. This regulation is transcriptional as well as post-transcriptional because overexpression of dMyc reduced the levels of *yki* mRNA and also reduced the levels of Yki protein when expressed from a tubulin promoter. dMyc thus regulates the activity of Yki in a negative feedback loop that keeps the activity of Yki and consequently dMyc in balance. This provides a homeostatic mechanism that is critical for the precise regulation of imaginal disc growth (Neto-Silva et al. 2010).

16.4 Cell Competition Induced by Defects in Cell Polarity

Cell competition is also engaged when epithelial cells suffer defects in apical–basal cell polarity. Apical–basal cell polarity in epithelial tissues is characterized by the asymmetrical morphology and localization of cellular components along the apical–basal axis and defects in cell polarity are often associated with cancer (reviewed in Dow et al. 2007; Elsum et al. 2012; Humbert et al. 2008; Vaccari and Bilder 2009). Apical–basal cell polarity is essential for proper cell proliferation, cell differentiation and tissue architecture, and its formation and maintenance is governed by the concerted action of three conserved protein complexes: the Crumbs (Crb), atypical Protein Kinase C (aPKC), and Scribble (Scrib) polarity modules (reviewed in Assemat et al. 2008; Dow et al. 2007; Humbert et al. 2008; Tepass et al. 2001). The Crb complex, composed of Crb, Patj, and Stardust (Sdt), and the aPKC complex, composed of aPKC, Par6, and Bazooka (Baz), localize to the subapical region of the plasma membrane and are important for the establishment and maintenance of the apical domain. The Scrib module contains Scrib, Discs large (Dlg), and Lethal giant larvae (Lgl), and is localized in the basolateral region. Notably, imaginal discs that are homozygous mutant for *scrib*, *dlg*, or *lgl* grow into large tumorous masses of neoplastic cells that display several hallmarks of carcinomas: They lose apical–basal cell polarity, they hyperproliferate, and they have defects in differentiation (reviewed in Elsum et al. 2012; Hariharan and Bilder 2006; Vaccari and Bilder 2009). Therefore, *scrib*, *dlg*, and *lgl* are called "neoplastic tumor suppressor genes."

Interestingly, however, the neoplastic phenotype of *scrib*$^-$ cells depends on their cellular environment. When *scrib*$^-$ cells are produced in clones of mutant cells that are surrounded by normal cells, they do not hyperproliferate but form small clones and are eliminated (Brumby and Richardson 2003; Doggett et al. 2011; Igaki et al. 2006, 2009; Leong et al. 2009; Ohsawa et al. 2011; Pagliarini and Xu 2003; Uhlirova et al. 2005). The requirement of normal cells for the elimination of tumorigenic *scrib*$^-$ clones is also demonstrated by the observation that genetically ablating the normal tissue surrounding *scrib*$^-$ cells results in hyperproliferation of the *scrib*$^-$ cells (Brumby and Richardson 2003; Igaki et al. 2009). More strikingly, reducing the fitness of surrounding cells by making them $M^{+/-}$, also allows the *scrib*$^-$ cells to hyperproliferate (Chen et al. 2012a). The suppression of the tumorigenic phenotype of *scrib*$^-$ cells thus depends on the fitness of their neighbors. Similar effects are also observed for *lgl*$^-$ and *dlg*$^-$ clones, although *lgl*$^-$ clones are eliminated more slowly and thus less efficiently (Froldi et al. 2010; Menendez et al. 2010; Ohsawa et al. 2011). In eye discs, *lgl*$^-$ clones do not lose cell polarity and cell competition of *lgl*$^-$ clones is weak (Grzeschik et al. 2010b), while in wing discs they eventually lose polarity and are outcompeted most strongly in the central region (Froldi et al. 2010). The conclusion from these experiments is that the presence of wild-type cells prevents *scrib*$^-$, *lgl*$^-$, and *dlg*$^-$ cells from manifesting their tumorigenic potential (Brumby and Richardson 2003; Chen et al. 2012a; Doggett et al. 2011; Froldi et al. 2010; Igaki et al. 2006, 2009; Leong et al. 2009; Menendez et al. 2010; Pagliarini and Xu 2003; Uhlirova et al. 2005). The elimination of *scrib*$^-$, *lgl*$^-$, and *dlg*$^-$ clones

thus fits the concept of cell competition, and such clones may have lower fitness than normal cells with respect to competitive behavior.

Similar to the rescue of $M^{+/-}$ cells from cell competition, loss of Hippo pathway activity or overexpression of Yki or dMyc is sufficient to rescue *lgl* and *scrib* mutant cell clones from being eliminated which then form tumorous masses instead (Chen et al. 2012a; Froldi et al. 2010; Menendez et al. 2010). The interpretation of this rescue is that hyperactivation of Yki or dMyc cell autonomously increases the fitness of mutant cells such that they can resist the competitive pressure from wild-type neighbors. However, an interesting twist to this effect was offered with the idea that hyperactivation of Yki may not only increase the survival rate of *lgl* mutant cells by directly promoting their survival, proliferation, and fitness but also because enhanced survival leads to more frequent merging of *lgl* mutant clones which then form larger aggregates providing a microenvironment in which cells can evade competitive interaction with wild-type cells (Menendez et al. 2010). In any case, the rescue of *lgl* and *scrib* mutant cell clones from elimination by hyperactivation of Yki provides further evidence that the Hippo pathway regulates competitive fitness.

16.5 Polarity-Dependent Cell Competition Regulates the Activity of the Hippo Pathway

The experiments with *scrib*$^-$, *lgl*$^-$, and *dlg*$^-$ clones showed that such clones are prevented from hyperproliferation and are eventually eliminated depending on the presence of wild-type cells. But how do wild-type cells cause this effect? Several groups found that the Jun N-terminal kinase (JNK) signaling pathway is activated in *scrib*$^-$ and *lgl*$^-$ clones, leading to engulfment and death or extrusion of mutant cells from the epithelium (Brumby and Richardson 2003; Cordero et al. 2010; Froldi et al. 2010; Igaki et al. 2009; Ohsawa et al. 2011; Uhlirova et al. 2005; Vidal 2010). The JNK pathway is an MAPKinase stress response pathway that is activated in response to various cellular stresses and can be sufficient to induce apoptosis (reviewed in Igaki 2009; Vidal 2010). Activation of JNK is required for the elimination of *scrib*$^-$ cells because blocking JNK activity in *scrib*$^-$ cells prevents their death and results in massive overgrowth of clones that is reminiscent of the tumorous overgrowth of entirely mutant discs (Brumby and Richardson 2003; Chen et al. 2012a; Cordero et al. 2010; Doggett et al. 2011; Igaki et al. 2009; Leong et al. 2009; Uhlirova et al. 2005). Similarly, blocking JNK in *lgl*$^-$ clones in eye discs results in loss of polarity and clonal overgrowth (Grzeschik et al. 2010b). However, blocking apoptosis directly by overexpression of the anti-apoptotic proteins p35 or DIAP1 did not cause overproliferation of *scrib*$^-$ (Brumby and Richardson 2003; Igaki et al. 2009) or *lgl*$^-$ clones (Grzeschik et al. 2010b). Therefore, in addition to inducing apoptosis, JNK suppresses the potential of *scrib*$^-$ cells to hyperproliferate (Brumby and Richardson 2003; Igaki et al. 2009), a process that involves regulation of the Hippo pathway.

When imaginal discs are entirely mutant for *scrib*, or when *scrib*$^-$ clones do not face cell competition because surrounding cells are $M^{+/-}$, then the *scrib*$^-$ cells hyperactivate Yki, which drives overgrowth of the mutant tissues into large neoplastic masses

(Chen et al. 2012a; Doggett et al. 2011; Grzeschik et al. 2010a). This result shows that normal cell polarity is essential for proper functioning of the Hippo pathway. In contrast, however, when *scrib*$^{-}$ cells face cell competition due to the presence of wild-type cells, then the *scrib*$^{-}$ cells do not hyperactivate Yki and the tumorigenic potential is suppressed (Chen et al. 2012a). The suppression of Yki activation is critical for the elimination of *scrib*$^{-}$ clones by cell competition, because experimental elevation of Yki activity in *scrib*$^{-}$ cells is sufficient to rescue them from cell competition and to fuel their neoplastic growth. Thus, cell competition between *scrib*$^{-}$ and wild-type cells acts as a tumor-suppression mechanism by regulating the activity of Yki in *scrib*$^{-}$ cells (Chen et al. 2012a).

In line with an effect on Yki, the levels of dMyc expression are critical for determining the fate of *lgl* mutant clones (Froldi et al. 2010; Menendez et al. 2010). *lgl* mutant clones that are generated in a wild-type background have low levels of dMyc expression, grow poorly, and are outcompeted. This competitive effect is strongest in the central part of the wing disc, the "wing pouch," which has the highest levels of endogenous dMyc expression. In contrast, *lgl* mutant clones that are generated next to *M*$^{+/-}$ mutant cells can overgrow and form tumorous masses with high dMyc expression. In addition, overexpressing dMyc rescues *lgl* mutant cells from elimination and promotes tumorous growth of *lgl* mutant cells. Thus, cell competition in this case prevents the elevation of dMyc expression and artificial overexpression of dMyc is sufficient to counteract the effects of cell competition. Since dMyc is a direct target of the Hippo pathway, this further supports that polarity defect-induced cell competition affects the activity of the Hippo pathway effector Yki. Yki may however not be a general target of cell competition, as Yki activity readouts were not affected in *M*-induced cell competition (Chen et al. 2012a; Martin et al. 2009).

Similar to overexpression of dMyc, overexpression of oncogenic Ras^{V12} is sufficient to rescue *lgl*$^{-}$ and *scrib*$^{-}$ clones from elimination by cell competition and double mutant cells form large and invasive tumors (Brumby and Richardson 2003; Chen et al. 2012a; Menendez et al. 2010). Such *lgl*$^{-}$+Ras^{V12} or *scrib*$^{-}$+Ras^{V12} tumors have a proliferation advantage and have high levels of nuclear Yki and Yki activity (Chen et al. 2012a; Menendez et al. 2010). Overexpressing Ras^{V12} in otherwise normal cells promotes competitive fitness and causes an upregulation of dMyc levels, while *ras* mutant clones have lower levels of dMyc, grow poorly, and are outcompeted (Prober and Edgar 2002). Ras may thus promote competitive fitness through regulating the levels of dMyc. This regulation of dMyc is probably independent of the Hippo pathway, because Ras^{V12} upregulated dMyc post-transcriptionally (Prober and Edgar 2002) and because Ras^{V12} expression only occasionally hyperactivated Yki (Chen et al. 2012a). Thus, the most likely scenario is that Ras^{V12} and dMyc overexpression rescue *lgl*$^{-}$ and *scrib*$^{-}$ cells from cell competition by promoting cellular fitness, which then negates the suppressive effect of cell competition on Yki activity resulting in the hyperactivation of Yki due to loss of cell polarity. Yki then in turn drives further expression of dMyc making *lgl*$^{-}$ and *scrib*$^{-}$ cells even more competitive and aggressive.

In summary, the findings discussed in the above sections show that the Hippo pathway regulates the competitive fitness of cells and that the activity of Yki itself is regulated by cell competition, at least in the case of cell polarity defects induced cell competition.

16.6 JNK Signaling and Molecular Mechanisms of Cell Competition

The JNK signaling pathway has prominent roles in cell competition and it has also been implicated in the regulation of the Hippo pathway in several different contexts. Interestingly, however, JNK signaling is required in winner and loser cells but for different effects and it may also play different roles depending on the cell competition assay.

JNK is often activated in loser cells such as $M^{+/-}$, *scrib*$^{-}$, *dlg*$^{-}$, *lgl*$^{-}$, and *tkv*$^{-}$ cells and blocking JNK signaling inhibits the death and prevents the elimination of such loser cells (reviewed in Tamori and Deng 2011; Igaki 2009; Vidal 2010). Because artificial activation of JNK signaling can be sufficient to induce apoptosis, it has been proposed that JNK activation causes the elimination of loser cells by inducing apoptosis (Moreno et al. 2002). However, the role of JNK signaling in loser cells is controversial. Indeed, several lines of evidence argue against a simple model in which JNK activation is required and sufficient to explain the elimination of loser cells. First, while there is evidence that JNK is required to eliminate $M^{+/-}$ loser cells (Moreno et al. 2002), others found that JNK was not required and argued that the requirement reported in the first study was due to the way mutant clones were induced (Tyler et al. 2007). Second, JNK is not required for the elimination of wild-type loser cells upon cell competition induced by dMyc overexpression (de la Cova et al. 2004). Third, JNK is often activated throughout clones of loser cells, but cell death is observed mainly at clone borders where loser cells are juxtaposed to competing winner cells. It is difficult to reconcile these findings with the simple model that JNK signaling is required and sufficient to induce apoptosis in loser cells. Thus, other signaling mechanisms between winner and loser cells must exist that cooperate with or that act independently of JNK signaling to eliminate loser cells.

Nevertheless, JNK signaling is clearly required in some loser cells, such as *scrib* mutant cells, for their elimination. One model to explain activation of JNK in loser cells is based on the idea that cells are competing for Decapentaplegic (Dpp), a secreted signaling ligand that promotes cell survival and tissue growth (Moreno et al. 2002). In this model, if cells are better at capturing Dpp, they gain a growth advantage and become winner cells. This Dpp competition model is based on the finding that $M^{+/-}$ cells have lower Dpp signaling activity, which in turn activates the JNK pathway to trigger apoptosis (Moreno et al. 2002). Supporting this model, increasing endocytosis in loser cells by overexpression of Rab5 prevented their elimination (Moreno et al. 2002). However, not all cell competition-inducing genotypes affect Dpp signaling (de la Cova et al. 2004); loss of Hippo signaling can rescue $M^{+/-}$ loser cells even in the absence of Dpp signaling (Tyler et al. 2007), and low levels of Dpp signaling alone cannot be sufficient to cause elimination because lateral regions of wing imaginal discs have low levels of Dpp signaling under normal conditions (reviewed in Neto-Silva et al. 2009). An alternative model to explain the activation of JNK in loser cells is based on the finding that clones of *scrib* mutant cell have elevated levels of endocytosis, which causes increased internalization and activation of Eiger, the *Drosophila* TNF homolog, which activates the JNK pathway

(Igaki et al. 2009). In this case, blocking endocytosis by overexpressing a dominant-negative form of Rab5 in *scrib* mutant cells blocked JNK activation and rescued *scrib* mutant cells from elimination. In addition, activation of JNK signaling in *scrib* mutant cells involves hemocytes, which are recruited to the site of mutant clones and secrete Eiger (Cordero et al. 2010). Altogether, it therefore appears that the activation of JNK in $M^{+/-}$ and *scrib* mutant clones involves different and potentially multiple mechanisms. Therefore, the role of JNK signaling in loser cells and the mechanism of its activation may be different depending on the nature (genotype) of the loser and winner cells.

Interestingly, JNK activation is not only observed in loser cells but sometimes also in winner cells. Upon the overexpression of dMyc, JNK is induced in the winner cells rather than wild-type loser cells (de la Cova et al. 2004). Nevertheless, removing JNK in the background does not affect dMyc-induced cell competition. In addition, JNK is activated in winner cells that are right next to loser cells with polarity defects, such as $scrib^{-}$, lgl^{-}, or dlg^{-} cells (Ohsawa et al. 2011). In these winner wild-type cells, JNK signaling induces expression of PVR, the *Drosophila* homolog of the PDGF/VEGF receptor, which in turn activates a phagocytic pathway that is mediated by ELMO (Engulfment and cell motility, Ced12 homolog) and Mbc (Myoblast city, a Ced5/DOCK180 homolog) (Ohsawa et al. 2011). Therefore, JNK signaling in winner cells induces them to engulf and phagocytose loser cells aiding in their elimination. However, this JNK–PVR engulfment pathway was not required for the elimination of $M^{+/-}$ cells and may thus be specific for the elimination of cells with defects in polarity (Ohsawa et al. 2011).

JNK signaling also affects the activity of Yki in ways that are context-dependent. During the elimination of $scrib^{-}$ mutant cell clones, JNK activation suppresses Yki activity (Chen et al. 2012a; Doggett et al. 2011). By contrast, during regeneration of wing imaginal discs that suffered damage from induced apoptosis, Yki is activated at wound sites and this activation requires JNK activity. In fact, low levels of JNK activation are sufficient to increase Yki activity (Sun and Irvine 2011). JNK can directly phosphorylate Yap1 (at sites that are different from the Lats1/2 phosphorylation sites) to regulate apoptosis in mammalian cells (Danovi et al. 2008; Tomlinson et al. 2010). However, it is not known whether the same sites also act to suppress the activity of Yki in other contexts. How JNK causes these various effects on Yki activity is thus not known and JNK likely cooperates with other signaling events to generate a specific response in order to cause its plethora of divergent effects.

16.7 Cell-to-Cell Signaling Mechanisms in Cell Competition

A central question in understanding cell competition is the question of how cells are designated to be losers or winners. Because loser and winner are not absolute properties but depend on the cellular microenvironment, these mechanisms may involve some kind of cell-to-cell signaling mechanisms. However, how cells sense differences in competitive fitness such as differences in M or dMyc activity is not known.

As discussed above, one model proposes that cells with lower levels of M or dMyc activity become losers because they have reduced levels of Dpp signaling compared to their neighbors (Moreno et al. 2002). However, this model still requires a mechanism whereby cells monitor and react to the relative Dpp signaling levels compared with their neighbors. There is evidence from experiments in *Drosophila* tissue culture that cell competition involves soluble factors, but such factors have not yet been identified (Senoo-Matsuda and Johnston 2007). Cell competition may act over a distance of several cell diameters in imaginal discs (de la Cova et al. 2004), but the induction of apoptosis is mostly relatively short range and occurs preferentially at boundaries of loser and winner cell populations (de la Cova et al. 2004; Li and Baker 2007; Moreno and Basler 2004; Moreno et al. 2002; Tyler et al. 2007). Recently, a number of different cell surface and extracellular signaling molecules have been implicated in cell competition.

Several genes were identified that are transcriptionally upregulated in loser cells generated upon cell competition induced by dMyc overexpression in imaginal discs. One of these genes, *flower* (*fwe*), is a three-pass transmembrane Ca^{2+} channel associated with neuronal synapses, but also expressed in imaginal discs cells, that has three alternatively spliced isoforms: Fwe^{ubi}, Fwe^{loseA}, and Fwe^{loseB} (Rhiner et al. 2010; Yao et al. 2009). These isoforms have the same transmembrane domains but differ in their extracellular C-termini. The Fwe^{ubi} isoform is ubiquitously expressed, but the Fwe^{lose} isoforms are expressed specifically in loser cells by $M^{+/-}$, *dMyc*, *scrib*$^{-}$, or *tkv*$^{-}$ (the *Drosophila* Dpp/BMP receptor homolog)-induced cell competition. Knockdown of the Fwe^{lose} isoforms blocks elimination of loser cells while clonal overexpression thereof induces apoptosis of normal cells. Significantly, uniform overexpression of any isoform did not cause apoptosis indicating that it is the relative level of Fwe isoforms that regulates cell survival. These studies thus identify Fwe as an extracellular signal by which cells are labeled as losers resulting in their elimination. However, the alternative splicing of Fwe is downstream from the determination of loser status and it will thus be interesting to elucidate the upstream signals that trigger the alternative splicing of Fwe. Interestingly, expression of the single mouse Fwe homolog *mFwe* was increased in and around DMBA/TPA-induced skin papillomas and homozygous mutant *fwe* mice developed fewer papillomas although they were otherwise normal (Petrova et al. 2012). Skin papillomas may thus use mFwe to promote their proliferation. Clonal expression of mFwe induced cell competition in *Drosophila* wing discs and mFwe may play an evolutionarily conserved role in cell competition (Petrova et al. 2012).

The same study also identified dSparc, the *Drosophila* homolog of the SPARC/Osteonectin-secreted glycoprotein to be induced in loser cells (Portela et al. 2010). Unlike Fwe, dSparc is however not required for the elimination of loser cells but acts to temporarily protect loser cells from apoptosis. This may give damaged cells a chance to recover, but if they are too impaired and cannot recover quickly then they are eliminated. SPARC is an ECM protein that modulates the activity of several growth factors and is involved in tumor development and tumor–stromal cell interactions, although its function is controversial and poorly understood (Clark and Sage 2008). The discovery that dSparc is involved in cell competition provides new avenues to study the role of this interesting protein family.

The transmembrane protein Crb localizes to the subapical plasma membrane domain just above adherens junctions and functions as a determinant for apical–basal cell polarity and as a receptor for the Hippo pathway (Chen et al. 2010; Grzeschik et al. 2010a; Ling et al. 2010; Robinson et al. 2010). It performs its function in the Hippo pathway by directly binding to and recruiting the Expanded (Ex) protein, a FERM domain containing adaptor protein that functions upstream in the Hippo pathway, to the plasma membrane. In *crb* mutant cells, Ex is mislocalized and gets degraded, Hippo pathway activity is compromised and Yki becomes hyperactivated (Chen et al. 2010; Ling et al. 2010). *crb* mutant cells thus induce Yki target genes and behave as super-competitors (Chen et al. 2010; Hafezi et al. 2012; Ling et al. 2010) which leads to the death of neighboring wild-type cells (Hafezi et al. 2012). Broad overexpression of Crb can phenocopy the *crb* loss of function phenotype, resulting in loss of Ex from the membrane, Yki hyperactivation and overgrowth, possible through dominant negative effects (Chen et al. 2010; Grzeschik et al. 2010a; Robinson et al. 2010). Interestingly, however, overexpression of Crb in clones of cells induces apoptosis, which was induced mainly along clone borders indicating that an interaction between Crb overexpressing cells with normal neighbors is essential for the induction of cell death (Hafezi et al. 2012). Moreover, cell death was induced in Crb overexpressing cells and in neighboring wild-type cells. Thus, Crb-mediated cell–cell interactions are important for the induction of apoptosis. Crb can recruit Crb molecules on neighboring cells to the cell–cell interface, thereby affecting Ex localization and Hippo signaling non-cell autonomously (Chen et al. 2010; Hafezi et al. 2012; Tanentzapf et al. 2000). Crb may thus affect cell survival by regulating the Hippo pathway cell autonomously and non-autonomously. However, whether all of the effects of Crb on cell survival depend on Hippo signaling, and how cells sense disparities of Crb levels between themselves and their neighbors is not known. One interesting possibility is that cells sense intracellular asymmetries in Crb localization, similar to other Hippo pathway components such as Fat, Dachsous (Ds), and Dachs (D) (Bosveld et al. 2012; Mao et al. 2006, 2011). Crb may therefore be part of a surveillance mechanism that induces apoptosis at the borders of different cell populations. It will be interesting to further investigate the role of Crb in mediating cell competition and other cell–cell surveillance mechanisms.

16.8 Cell Competition in Mammalian Systems

Phenomena that are similar to cell competition in *Drosophila* have also been reported in mammals. Like *M* mutations in *Drosophila*, a mutation in the mouse ribosomal protein RpL24 causes a dominant slow growth phenotype and *RpL24* heterozygous cells are outcompeted in chimeras with normal cells (Oliver et al. 2004). Cell competition is occurring during liver cell repopulation in rats (Oertel et al. 2006): After partial hepatectomy, transplanted fetal liver stem/progenitor cells proliferated and repopulated normal adult livers by progressive replacement of the less proliferative host hepatocytes through a mechanism that involved the induction of apoptosis of

host cells immediately adjacent to transplanted cells. The expansion of the transplanted cell population at the expense of the host cells thus resembles the effects of cell competition in *Drosophila* imaginal discs. Another striking example of cell competition is presented by the competition between hematopoietic stem and progenitor cells (Bondar and Medzhitov 2010). There, cell competition resulted in the growth arrest of cells that suffered stress, which was dependent on the relative levels of p53.

Cell competition is also observed in mammalian tissue culture, where several tumor suppressors and oncogenes known to affect cell competition in *Drosophila* have been found to affect cellular behavior in mixed cell populations. Loss of the single Scrib homolog in Madin-Darby Canine Kidney (MDCK) cells induced apoptosis due to cell competition (Norman et al. 2012). Notably, apoptosis of *scrib* knockdown cells was only observed when they were surrounded by normal cells but not when they were cultured among themselves. *Scrib* knockdown cells that were next to wild-type cells were apically extruded from the epithelium, underwent apoptosis and were eventually eliminated from the culture (Norman et al. 2012). Therefore the elimination of the *scrib* knockdown cells thus depends on interaction with surrounding normal cells, similar to the elimination of *scrib* mutant cell clones in *Drosophila* imaginal discs. However, whether this elimination involves regulation of the mammalian Yki homologs Yap or Taz is not known, but resembling the situation in *Drosophila* it required a mitogen-activated protein kinase (MAPK) stress pathway, in this case the p38 MAPK.

Similar to Scrib, knockdown of the Lgl1/2 binding protein Mahjong (Mahj, also known as $CRL4^{DCAF1}$ and Vpr-BP) in MDCK cells induced elimination of mutant cells that was only observed when mutant cells were cocultured with normal cells (Tamori et al. 2010). Likewise, clones of imaginal disc cells mutant for the *Drosophila mahj* homolog were eliminated by cell competition similar to *lgl* mutant clones. *mahj* mutant clones, however, did not show polarity defects and Mahj overexpression rescued lgl^- but not $scrib^-$ clones from cell competition. Mahj may thus specifically act downstream of Lgl in cell competition. Mahj is an adaptor protein that recruits Merlin, a Hippo pathway component encoded by the Neurofibromatosis 2 (NF2) tumor suppressor gene, to the Cullin4A E3 ubiquitin ligase complex and targets it for degradation (Huang and Chen 2008). In addition, Mahj functions downstream of Merlin (Cooper et al. 2011; Li et al. 2010). It is therefore possible that Mahj functions in cell competition through regulation of the Hippo pathway. Altogether, cell competition induced by polarity defects may be conserved between *Drosophila* and mammals and potentially serve as a surveillance mechanism to eliminate tumorigenic cells in mammalian epithelia.

16.9 Perspectives

The currently postulated role of cell competition is as a homeostatic mechanism during development and homeostasis where the fitness of a tissue is maximized by eliminating abnormal cells. In this role, cell competition may act as a tumor suppressor mechanism to eliminate tumorigenic cells. The elimination of cells with defects in

epithelial cell polarity from growing *Drosophila* imaginal discs and from cultured MDCK cell sheets is a dramatic example of this function. The homeostatic model of cell competition is supported by experiments from *Drosophila* and mammals and many of the same genetic aberrations in tumor suppressors and oncogenes cause cell competition. Cell competition may therefore involve conserved mechanisms.

Cell competition may have a critical function in cancer biology not only as a tumor suppression mechanism that eliminates precancerous cells but also as a mechanism that promotes the expansion of a population of precancerous cells (Baker and Li 2008; de Beco et al. 2012; Moreno 2008; Petrova et al. 2011; Rhiner and Moreno 2009). Overexpression and hyperactivation of the oncogenes Myc, Ras^{V12}, and YAP/TAZ are frequently observed in many different types of tumors and their hyperactivation directly drives cell growth and proliferation. These genes may, however, drive cancer development also by transforming the mutant cells into supercompetitors. Like in *Drosophila*, hyperactivation of Myc, Ras^{V12}, and YAP/TAZ may drive the clonal expansion of mutant cells at the expense of neighboring wild-type cells and may also protect mutant cells from competitive cell–cell interactions that would otherwise eliminate abnormal cells. Understanding the mechanisms of cell competition may thus help understand how precancerous lesions survive and evolve into cancer. Cell competition may also be involved in the elimination of metastatic cells, which may need to evade cell competition to survive. The Hippo pathway and other oncogenic pathways may therefore contribute to cancer development first by their direct effects on cell proliferation and second by their effects on cell competition. However, whether Myc, Ras^{V12}, and YAP/TAZ indeed drive cell competition in mammals is not known but an important area for future studies. Investigating how the Hippo pathway functions in different cell competition mechanisms may shed light onto early stages of tumor development and may provide novel strategies for cancer treatment.

Our understanding of the molecular mechanisms that operate in cell competition is rudimentary and many questions remain. For example, we still do not know what biological properties determine cell fitness and how cells sense and compare their fitness with that of their neighbors. Thus we do not know how cells interact to determine losers and winners. It appears that different mechanisms may be employed under different competitive situations such as $M^{+/-}$or *dMyc*-driven cell competition as opposed to cell competition triggered by loss of cell polarity. The discovery of the molecules and pathways described in this review provide important new inroads into elucidating the mechanisms of cell competition but these pathways are only the beginning of understanding cell competition. Given all the new molecules and surprises that cell competition research has already uncovered, we can expect a continuingly exciting journey.

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Chapter 17
Non-canonical Roles for the Hippo Pathway

Xaralabos Varelas

Abstract The Hippo pathway is a conserved signaling pathway that mediates tissue and organ growth, and is commonly deregulated in diseases such as cancer. A series of phosphorylation events and protein interactions propagate Hippo pathway signaling and culminate in the modulation of the transcriptional regulators TAZ and YAP. The majority of effort to date has focused on identifying new players in the pathway and understanding the transcriptional processes regulated by TAZ and YAP. However, it has become clear that the ramifications of Hippo pathway activity extend well beyond that of transcriptional regulation. Hippo pathway components have been implicated in a wide-range of processes that range from the organization of cell polarity to the fine-tuning of morphogen signaling. This chapter highlights some of the non-canonical roles for the Hippo pathway and discusses how these functions influence organogenesis and disease.

Keywords TAZ • YAP • Hippo • Cell cycle • Apoptosis • Polarity • Cilia • PCP • Wnt • TGFβ • Notch • Ubiquitin

17.1 Introduction

The accurate development of tissues and organs requires the coordination of cell division, cell death, and cell fate. How such events are interconnected has remained a central question in biology for decades. The discovery and delineation of the Hippo pathway has shed light on how these events are organized and contribute to organ growth. The Hippo pathway serves as an important conduit by which extracellular cues are interpreted and transmitted intracellularly, ultimately converging

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0_17,

on transcriptional regulation. However, as our understanding of the Hippo pathway develops, it is becoming clear that the pathway has multifaceted functions that extend well beyond linear signals that control transcription.

The core Hippo signaling pathway components were first delineated in *Drosophila melanogaster* from screening approaches aimed at identifying mutations that promote tissue overgrowth (for reviews, see Pan 2010; Harvey and Tapon 2007). Genetic epistasis experiments, in addition to biochemical analysis, ordered the pathway as a series of phosphorylation events mediated by the Hippo (Hpo) and Warts (Wts) kinases that ultimately control the localization and activity of the transcriptional regulator Yorkie (Yki). The evolutionary conservation was later described, and its importance in mammalian development and disease has become prominent. Central to the mammalian kinase cascade are the NDR (nuclear Dbf2-related) family kinases LATS1 and LATS2 (LATS1/2), which receive inputs from a number of upstream regulatory proteins (Fig. 17.1) (for reviews, see Pan 2010; Zhao et al. 2011b). LATS1/2 phosphorylate the transcriptional regulators TAZ and YAP (TAZ/YAP). Hypo-phosphorylated TAZ/YAP localize to the nucleus where they interact with transcription factors to direct key transcriptional events. Deregulated nuclear TAZ/YAP activity strongly promotes tumorigenesis in most mammalian model systems studied (Pan 2010; Zhao et al. 2011b). Thus, as in flies, Hippo pathway-mediated regulation of transcription in mammals is crucial for restricting tissue growth. Consequently, much of the focus on TAZ/YAP has been on understanding their nuclear roles. Several studies however have revealed important non-transcriptional roles for Hippo pathway effectors, indicating that Hippo pathway activity is more complex than originally anticipated. For instance, components of the Hippo pathway have been implicated in directing protein ubiquitination, intracellular protein transport, cilia formation, cell cycle checkpoints, the regulation of cell polarity, and in the fine-tuning of developmental signals. This chapter will review and discuss some of the data describing these less-appreciated contributions of the pathway.

17.2 Non-canonical Roles for Hippo Pathway Kinases

While regulation of TAZ/YAP is important for relaying many processes directed by the MST and LATS kinases, data has indicated that LATS and MST function beyond TAZ/YAP (Fig. 17.2). In fact, long before their inauguration into the Hippo pathway, LATS and MST kinases were found to directly regulate several factors that are crucial for cell cycle and stress response. One of the first studies linking mammalian LATS kinases to cell cycle regulation described the ability of LATS1 to influence CDC2 activity (Tao et al. 1999). LATS kinases interact with CDC2, consequently preventing CDC2 activation by mitotic cyclins and thereby inhibiting cell cycle progression. LATS1 also directly inhibits the activity of LIMK1, a kinase that transmits signals from Rho-GTPases to modulate actin polymerization and cytokinesis (Yang et al. 2004). Additionally, LATS1/2 bind the mitotic-specific kinases Aurora A and Aurora B, and promote the phosphorylation of Aurora B, although this could

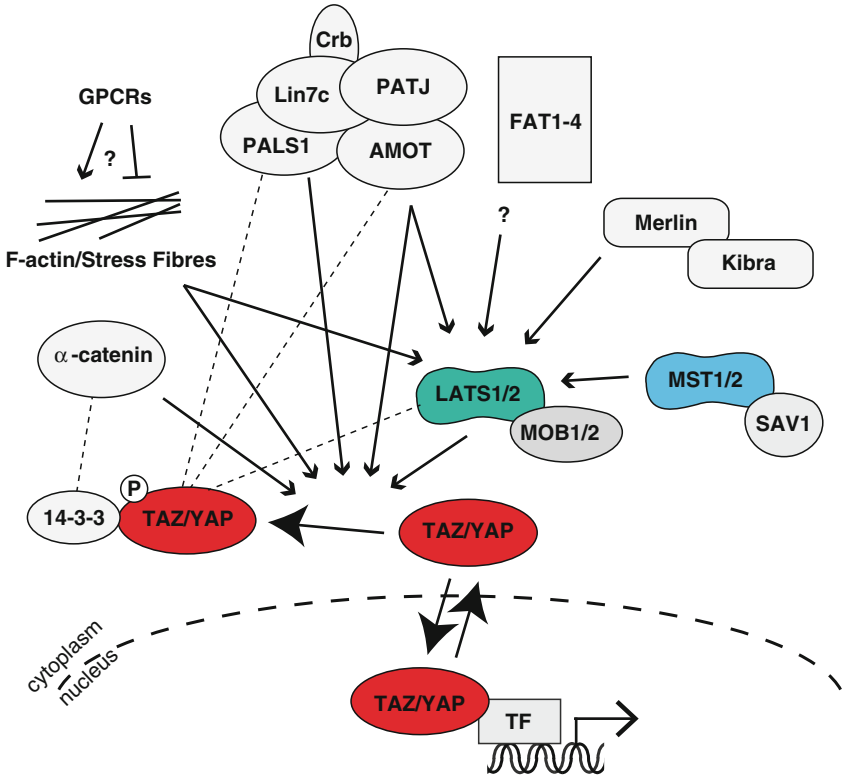

Fig. 17.1 The canonical Hippo pathway in mammals. Shown are elements that control the phosphorylation and localization of the transcriptional regulators TAZ and YAP (TAZ/YAP), including the core Hippo pathway kinases MST1/2 and LATS1/2. Many upstream regulators directly bind TAZ/YAP (*dotted lines*) and some may control TAZ/YAP phosphorylation and localization independently of the MST1/2 and LATS1/2 kinases. In the nucleus TAZ/YAP direct the activity of a range of transcription factors (TF), enabling the regulation of diverse biological processes

be indirect (Yabuta et al. 2011; Toji et al. 2004). Interestingly, a converse relationship also exists, as LATS2 is a direct target of Aurora A. Thus, the Aurora-LATS axis likely plays key roles in mitotic progression.

The Hippo kinase homolog MST1 also impacts Aurora B phosphorylation, specifically to promote accurate kinetochore-microtubule attachment and proper chromosome segregation (Oh et al. 2010). MST2 and the Hippo pathway adaptor protein SAV1 also influence chromosome segregation by directing Nek2A kinase to centrosomes (Mardin et al. 2010). Nek2A phosphorylates C-Nap1 and rootletin, which are part of a protein complex required for accurate centrosome cohesion, and this promotes their displacement from the centrosome. In a related function, MST2 and SAV1 also cooperate with Nek2A to control the kinesin motor Eg5 to enable centrosome separation and bipolar spindle formation (Mardin et al. 2010).

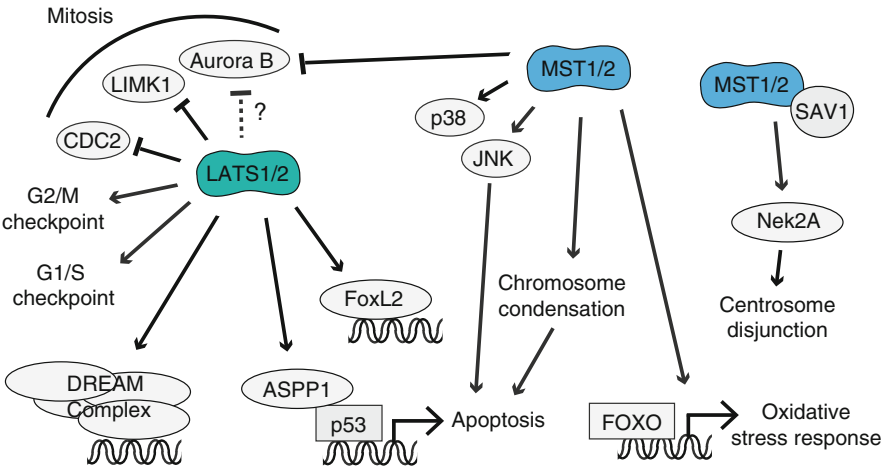

Fig. 17.2 Diverse signaling by MST and LATS kinases. Illustrated are the roles of the MST1/2 and LATS1/2 kinases that are thought to be independent from their influence on TAZ/YAP activity. As indicated, primarily roles for these kinases include the direct regulation of cell cycle and apoptotic effectors

Hippo pathway effectors also direct cell cycle progression by mediating cell cycle checkpoint fidelity. For example, LATS1 plays a critical role in activating the DNA damage checkpoint by phosphorylating MYPT1 (myosin phosphatase-targeting subunit 1), a regulatory subunit of the PP1C phosphatase (Chiyoda et al. 2012). LATS1-induced phosphorylation of MYPT1 directs PP1C activity toward PLK1 (polo-like kinase 1), thereby suppressing PLK1 activity. Activation of the DNA damage checkpoint relies on efficient PLK1 suppression, and thus LATS1-mediated regulation of MYPT1 makes an important contribution to this checkpoint.

LATS kinases additionally contribute to the G1/S checkpoint by binding and inhibiting Mdm2, an ubiquitin-ligase that targets p53 for degradation (Aylon et al. 2006). LATS2 also functions to direct p53-dependent apoptosis indirectly by promoting the nuclear accumulation of ASPP1 (apoptosis-stimulating protein of p53-1). In response to oncogenic stress LATS2 phosphorylates ASPP1 to drive ASPP1 nuclear accumulation and p53 recruitment to pro-apoptotic promoters (Aylon et al. 2010). Cytoplasmic ASPP1 (and ASPP2), on the other hand, disrupts LATS1 interaction with TAZ/YAP, thereby promoting TAZ/YAP nuclear localization (Vigneron et al. 2010). In the nucleus YAP prevents LATS2 association with ASPP1. Thus, the cytoplasmic-to-nuclear balance of ASPP1, LATS1/2, and TAZ/YAP is critical for controlling apoptosis both through canonical TAZ/YAP-mediated transcriptional regulation and non-canonical regulation of p53 activity. MST1 also plays an important role in activating the p53 apoptotic response, in this case by inactivating the Sirtuin1 deacetylase via phosphorylation, consequently enhancing p53 acetylation and activity (Yuan et al. 2011).

Mammalian MST1/2 kinases were initially identified based on their homology to the Ste20 kinase in yeast and classified as stress response kinases that promote

apoptosis (Taylor et al. 1996; Graves et al. 1998). MST1 relays apoptotic cues by activating JNK and p38 MAP kinases to promote caspase-3 activation (Graves et al. 1998; Ura et al. 2001). Furthermore, MST kinases stimulate the phosphorylation of histones H2AX and H2B to induce chromatin condensation and DNA fragmentation (Wen et al. 2010; Cheung et al. 2003; Ura et al. 2007). Interestingly, these mechanisms appear distinct from those regulating apoptosis through YAP-mediated transcription (Matallanas et al. 2007).

Several transcription factors unrelated to the canonical Hippo pathway have been identified as targets of the LATS and MST kinases. A noteworthy group regulated by MST kinases is the FOXO family of forkhead transcription factors, which control a variety of biological functions including oxidative stress resistance. MST1 phosphorylates FOXO proteins, which disrupt their ability to bind to 14-3-3 proteins, thereby promoting their nuclear localization and activity (Lehtinen et al. 2006). Knockdown of the MST1/2 homolog in *Caenorhabditis elegans* accelerates tissue aging and shortens life span, while MST overexpression has the opposite effect. This phenotype may be explained primarily by MST regulation of FOXO, since FOXO has been shown to affect life span in a broad range of evolutionary diverse organisms (Salih and Brunet 2008). Forkhead L2 (FoxL2), another forkhead transcription factor, is phosphorylated by LATS1. FoxL2 is critical for mammalian ovary development and the activity of this transcriptional repressor is activated by LATS1 phosphorylation (Pisarska et al. 2010). Another important transcriptional complex regulated by LATS kinases is the DREAM (DP, retinoblastoma [RB], E2F, and MuvB) repressor complex. LATS2 phosphorylates and activates DYRK1A, a kinase upstream of DREAM (Tschop et al. 2011). Knockdown of LATS2 suppresses RB-induced senescence, suggesting that LATS2 promotes RB activity through DYRK1A-mediated assembly of the DREAM complex. Given the importance of the Rb-E2F pathway in cell cycle progression and cell fate, this mode of regulation is likely a critical means by which LATS kinases can direct these events.

Together these studies provide ample evidence to indicate that the MST and LATS kinase functions extend well beyond merely regulating TAZ/YAP transcriptional activity. As noted, these roles include maintaining accurate chromosome segregation, cell cycle checkpoint activation, and transcriptional responses that are required for accurate cell cycle progression, as well as sensing environmental stress to induce apoptosis. Further work is required to pinpoint how these events specifically contribute to development and disease, but given their fundamental nature it is likely that these regulatory mechanisms will prove important.

17.3 The Regulation of Epithelial Architecture by the Hippo Pathway

In addition to the deregulation of cell proliferation and apoptosis, genetic aberrations in Hippo pathway also induce the rearrangement of epithelial architecture resulting in folded and darker mutant tissue, much like the hide of a hippopotamus

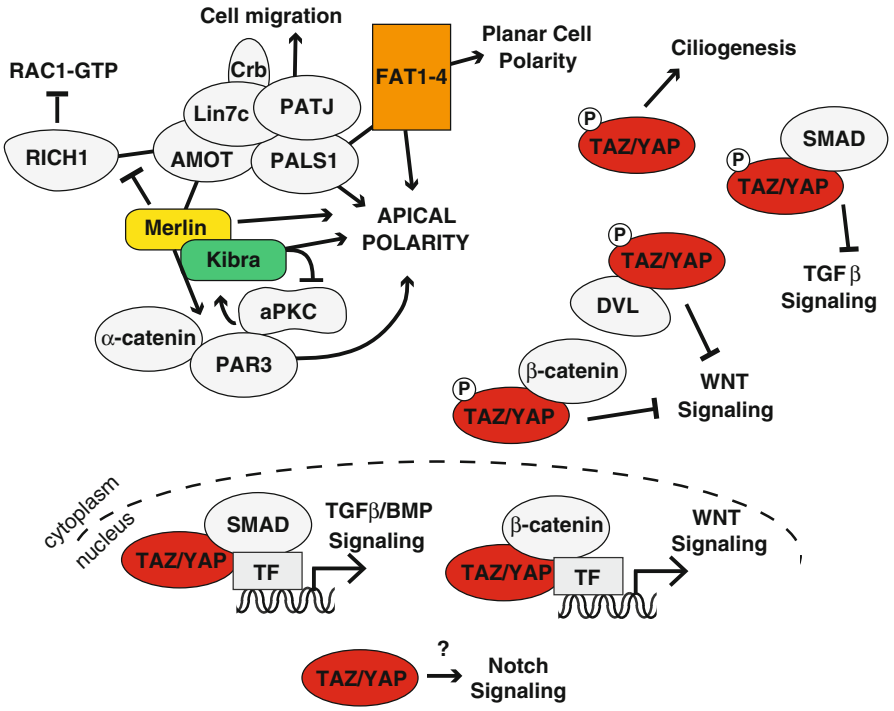

Fig. 17.3 Non-canonical roles for Hippo pathway effectors. Hippo pathway effectors influence a range of processes beyond cell proliferation and apoptosis, including directing apical–basal and planar cell polarity, ciliogenesis and morphogen signaling. Several of the proposed mechanisms linking the Hippo pathway to these events are highlighted

(hence the pathway name). Studies in flies have revealed that these functions are independently controlled from the growth-regulating function of the pathway and rather result from the abnormal localization and levels of proteins important for the control of cell polarity (polarity proteins) (Genevet et al. 2009; Hamaratoglu et al. 2009). While these polarity-related defects may in part be mediated by Yki/TAZ/YAP-directed transcription, interactions between Hippo pathway effectors and regulators of epithelial architecture suggest more direct roles for the Hippo pathway in the establishment and/or maintenance of cell polarity (Fig. 17.3).

Epithelial cells possess distinct apical, basal, and lateral domains (apical–basal polarity) (Assemat et al. 2008; Shin et al. 2006). This asymmetry is established by the distinct localization of a set of polarity protein complexes that are recruited and regulated by cell contact-induced tight and adherens junctions. The apical–epithelial domain is defined by two protein complexes: (1) the Crumbs (CRB) complex, which in mammals is composed of Pals1, PATJ, Mpdz, Lin7c, and the related Crumbs proteins (Crb1, Crb2, Crb3); and (2) the PAR complex, which is made up of Par3, Par6, and the atypical Protein Kinase C (aPKC) (Assemat et al. 2008). The basolateral domain is specified by the activity of the Scribble (SCRIB) complex, which is

composed of Scribble, Disc-large (Dlg), and lethal giant larvae (Lgl) (Assemat et al. 2008). Interestingly, many of the proteins that comprise these polarity complexes bind to Hippo pathway components.

The most extensively studied of the upstream Hippo pathway components with respect to its contribution to cell polarity is ostensibly Merlin (encoded by the *Nf2* gene). Merlin is a FERM (*F* for 4.1-*E*zrin-*R*adixin-*M*oesin) domain-containing protein that had been studied long before its involvement in Hippo signaling due to the association of mutations with type II neurofibromatosis, an inherited disease that results in the development of bilateral vestibular schwannomas (Zhou and Hanemann 2012). Genetic analysis in *D. melanogaster* initially linked Merlin to the Hippo pathway, placing Merlin upstream of the Hippo pathway kinase cascade (Hamaratoglu et al. 2006). Genetic studies in mice indicate conservation of Merlin function in Hippo signaling (Zhang et al. 2010; Liu-Chittenden et al. 2012), but imply tissue and context dependence (Benhamouche et al. 2010; Gladden et al. 2010). Merlin has an important role in stabilizing adherens junctions that, in part, involves directing interaction between the PAR complex component Par3 and the adherens junction-associated protein α-catenin (Lallemand et al. 2003; Gladden et al. 2010). $Nf2^{-/-}$ epidermal epithelial cells lack adherens junctions and display a disorganized cell polarity (Gladden et al. 2010). In a likely related role, Merlin associates with components of the CRB complex, including the CRB-associated AMOT proteins (Yi et al. 2011). AMOT proteins are recruited to tight junctions by the CRB complex as cells establish polarity and AMOT is capable of binding and inhibiting the Rho-GAP Rich1 subsequently impacting Cdc42 and Rac1 GTPase activity (Wells et al. 2006). Merlin has been shown to compete with Rich1 for AMOT, thereby generating free active Rich1 and indirectly promoting Rich1 GAP activity on Rac1 at tight junctions. (Yi et al. 2011). Intriguingly, many of the polarity proteins associated with Merlin also bind TAZ/YAP. For example, α-catenin has been shown to recruit 14-3-3 bound YAP, and the binding of TAZ/YAP to AMOT and CRB complex components is important for restricting nuclear TAZ/YAP activity (Chan et al. 2011; Schlegelmilch et al. 2011; Varelas et al. 2010b; Zhao et al. 2011a). Thus, the role of Merlin in directing cell junction formation and polarity may also be a mechanism by which Merlin facilitates Hippo pathway activity.

The Merlin-associated protein Kibra (also known as WWC1) also has key roles in regulating Hippo signaling and in establishing epithelial cell polarity. A role for Kibra in Hippo signaling was first identified in flies, which revealed that Kibra forms a complex with Merlin and another FERM domain protein called Expanded (Yu et al. 2010; Genevet et al. 2010; Baumgartner et al. 2010). This complex binds and activates the Hpo and Wts kinases, thereby promoting Yki phosphorylation. Human Kibra has a conserved role in activating the human LATS kinases, suggesting it is an important regulator in mammals as well (Yu et al. 2010; Xiao et al. 2011). Interestingly, Kibra was first identified as a protein important for human memory performance (Papassotiropoulos et al. 2006). Recent work shows that Kibra binds to the protein interacting with C-kinase 1 (PICK1) and forms a complex with α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs), the major excitatory neurotransmitter receptors in the brain (Makuch et al. 2011).

Given that PICK1 associates with PKC kinases (Staudinger et al. 1997), and moreover that Kibra is a substrate of PKCzeta (Buther et al. 2004) and regulates aPKC activity (Yoshihama et al. 2011), it is likely that this relationship between Kibra and PICK1 will prove to be important with regard to the transmission of polarity information.

Proper regulation of aPKC activity is critical for epithelial polarity (Shin et al. 2006), and Kibra can mediate epithelial polarization by binding and inhibiting aPKC kinase activity (Yoshihama et al. 2011). Like Merlin, Kibra is localized to adherens and tight junctions in epithelial cells, and knockdown of Kibra leads to an apical expansion of epithelial cells grown in three-dimensional cultures (Yoshihama et al. 2011). The expansion of the apical domain results from defective apical trafficking, in large part due to deregulated aPKC activity. Kibra also binds to PATJ, a component of the CRB complex (Duning et al. 2008). Interestingly, Kibra and PATJ co-localize to the leading edge of migrating podocytes, and knockdown of either Kibra or PATJ results in defective directional cell migration (Duning et al. 2008; Shin et al. 2007). PATJ–AMOT interaction alters the local activity of the GTPase RhoA in lamellopodia (Ernkvist et al. 2009), suggesting that Kibra, like Merlin, may influence AMOT function. These data point to central roles for these proteins in directing polarity required for cell migration, in addition to Hippo signaling and apical–basal–epithelial polarity.

The CRB complex protein Pals1 interacts with FAT4, a large atypical protocadherin homologous to Fat in *D. melanogaster*. The fly Fat cadherin is a key upstream regulator of Hippo signaling that forms hetero- and homophilic interactions with another proto-cadherin known as Dachsous (Dachsous 1 and 2 in mammals) (Clark et al. 1995). Dachsous binding promotes Fat phosphorylation and conjures cell contact signals (Sopko et al. 2009; Feng and Irvine 2009). The interaction between Pals1 and FAT4 has been suggested important for apical membrane organization of mouse neural progenitors given that depletion of FAT4 from neural progenitors of the mouse embryonic cerebral cortex leads to a loss of the apical domain in these cells. Dachsous1 is also involved in this apical polarity-organizing function, and is suggested to do so by promoting FAT4 activity (Ishiuchi et al. 2009). Fat and Dachsous cadherin interactions are best described in flies, where Fat-Dachsous binding dictates another form of polarity known as planar cell polarity (PCP) (Sopko and McNeill 2009; Thomas and Strutt 2012). However, whether this role in coordinating apical domain organization is linked to PCP is unclear.

17.4 The Role of the Hippo Pathway in Ciliogenesis and Planar Cell Polarity

Another emerging role for the Hippo pathway is in the regulation of primary cilia assembly. Primary cilia are specialized organelles that extend from the apical surface to relay signals from the luminal space (Goetz and Anderson 2010). Most mammalian cells possess cilia and these organelles have been implicated in mediating

mechanosensation. Directional bending of primary cilia impacts key developmental events, and defects in the regulation of cilia result in a range of developmental disorders (Goetz and Anderson 2010). Genetic studies have indicated that TAZ and YAP participate in cilia-regulated events, revealing a noteworthy and relatively unexplored relationship.

A connection between ciliogenesis and the Hippo pathway was first illuminated from the analysis of *Taz* knockout mice. Mice with a biallelic deletion of the *Taz* gene develop severe renal cysts that lead to end-stage polycystic kidney disease (Hossain et al. 2007; Makita et al. 2008; Tian et al. 2007). Cells lining the cysts in *Taz* knockout kidneys have fewer and shorter cilia, and siRNA-mediated knockdown of TAZ in vitro disrupts cilia length (Hossain et al. 2007). The expression of several genes associated with ciliogenesis is reduced in *Taz* knockout mice, and transcriptional alterations may explain these observations in part (Hossain et al. 2007). However, TAZ localizes to primary cilia (Hossain et al. 2007), suggesting a more direct role for TAZ in cilia development and/or maintenance. One role for TAZ in ciliogenesis may be in the regulation of polycystin 2 (PC2, encoded by the *Pkd2* gene), a nonselective calcium-permeable cation channel protein responsible for mediating the mechanosensing properties of cilia. TAZ interacts with PC2 and has been suggested to control the levels of PC2 by recruiting the β-TRCP/SCF ubiquitin-ligase complex and targeting PC2 for ubiquitin-mediated proteolysis (Tian et al. 2007). The NIMA-related kinase NEK1 phosphorylates Serine 309 in mouse TAZ (Serine 314 in human TAZ), a residue within a TAZ phosphodegron motif that recruits the β-TRCP/SCF ubiquitin-ligase complex. NEK1-mediated phosphorylation of Ser309 allows TAZ to recruit β-TRCP to ubiquitinate and degrade PC2 (Yim et al. 2011). The loss of PC2 does not affect cilia formation per se, but rather affects the ability of the primary cilium to function as a flow sensor (Nauli et al. 2003). Thus, regulation of PC2 may allow TAZ to direct mechanosensory cues.

Cilia are thought to function as signaling centers that impact on a number of pathways, including Hedgehog and Wnt (Goetz and Anderson 2010). TAZ and YAP may have a role in directing these signals at cilia. The transcription factor Glis3, a protein that localizes to cilia and has high sequence similarity to the Hedgehog-regulated Gli transcription factors, is regulated by TAZ (Kang et al. 2009). Glis3 recruits TAZ to the nucleus and Glis3 transcriptional activity depends on interaction with TAZ; therefore, the localization of these factors to the primary cilia may be one mode by which these organelles influence cell signaling. Primary cilia negatively regulate Wnt/β-catenin signals by mediating Dvl phosphorylation (Corbit et al. 2008). Non-nuclear TAZ also impinges Dvl phosphorylation to downregulate Wnt/β-catenin responses (Varelas et al. 2010a). It is therefore possible that cilia-localized TAZ may be involved in this regulation. Additionally, Dvl proteins play key roles in the apical docking and planar polarization of basal bodies, which affect cilia orientation (Park et al. 2008; Hashimoto et al. 2010). Thus, the relationship between TAZ and Dvl may influence primary cilia function. Other Hippo pathway effectors besides TAZ have been additionally linked to cilia-directed cues. For example, the cilia-associated protein NPHP4 associates with and negatively

regulates LATS2 activity (Habbig et al. 2011). In this manner, NPHP4 can alter the nuclear pool of TAZ and serve as a sensor to balance nuclear- vs. cilia-related TAZ functions.

Interestingly, *Fat4* knockout mice display cilia-related defects much like that observed for TAZ mutant mice (Saburi et al. 2008). Like TAZ, FAT4 localizes to primary cilia, and deletion of *Fat4* leads to defects in cilia formation (Saburi et al. 2008), although the effects on cilia are not as severe as those in *Taz* knockout mice. Interestingly, like its *D. melanogaster* counterpart, FAT4 has a key role in the regulation of PCP—a distinct form of cellular asymmetry that organizes cells perpendicular to the apical–basal plane of tissues (Saburi et al. 2008). Many developmental processes rely on accurate PCP for organized cell movements, for instance those required for shaping organs. The disruption of PCP leads to a range of defects, including convergent extension-mediated tube elongation problems that result in defects associated with neural tube closure, kidney tube elongation, and cochlear extension. *Fat4* knockout mice display phenotypes characteristic of defective PCP, and these phenotypes are strengthened when combined with the deletion of other PCP regulators such as Vangl2 (Saburi et al. 2008). Four versions of FAT exist in mammals (FAT1–4), and redundant functions are suggested (Saburi et al. 2012): PCP defects in *Fat1*/*Fat4* double mutant mice are strongly enhanced compared to *Fat1* knockout (Ciani et al. 2003) or *Fat4* knockout mice (Saburi et al. 2008). Genetic studies in flies indicate that the PCP and growth regulatory functions of Fat are separable, indicating that downstream effectors mediating these effects are likely different (Matakatsu and Blair 2012). Whether FAT cadherins signal through TAZ/YAP in mammals is unclear, particularly with respect to PCP. However, the cystic kidney phenotypes in TAZ knockout mice and the relationship between the Hippo pathway and cilia hint that TAZ and/or YAP may contribute directly to PCP.

17.5 Hippo at the Nexus of Signaling Crosstalk

A sophisticated network of signaling directs tissue and organ development and defects in the integration and coordination of individual signals is often the basis of disease. Recent studies have positioned the Hippo pathway at the nexus of signaling organization, directing cues initiated by other developmentally important pathways (Mauviel et al. 2012). This section highlights some of the roles described for Hippo pathway effectors that converge with other signaling pathways.

17.5.1 TGFβ Signaling

The TGFβ superfamily encompasses the TGFβ, BMP, activin and growth differentiation factors, all of which are secreted morphogens that activate a complex of serine/threonine kinase receptors (Attisano and Wrana 2002; Wu and Hill 2009).

Ligand–receptor interactions promote the phosphorylation of a class of transcription factors known as the receptor-regulated Smads (R-Smads), which subsequently accumulate in the nucleus and direct transcription. Several associations have been made between the Hippo pathway and the TGFβ family that range from the regulation of TGFβ receptor activation (Ferrigno et al. 2002) to the direct control of transcriptional events (Varelas and Wrana 2012). The Hippo pathway effectors TAZ/YAP are intimately involved in directing these roles, in large part by binding and directing the activity of TGFβ-regulated Smad2 and Smad3 (Smad2/3) and BMP-regulated Smad1 proteins (Varelas et al. 2008; Alarcon et al. 2009). In accordance, TAZ/YAP facilitate the response of Smads to TGFβ and BMP in scenarios where TAZ/YAP and Smad2/3 are both nuclear (Varelas et al. 2008; Alarcon et al. 2009).

TAZ/YAP are also implicated in regulating nuclear Smad protein stability. TAZ/YAP do so by binding and protecting Smads from ubiquitin-mediated degradation by Smurf1 and Nedd4L ubiquitin ligases (Alarcon et al. 2009; Aragon et al. 2011). This protective role for TAZ and YAP appears quite pervasive. For instance, YAP binds and protects p73 from ubiquitin-mediated degradation by the ubiquitin ligase Itch (Levy et al. 2007). Additionally, YAP binding blocks ubiquitination of Klf5 by the WWP1 ubiquitin ligase (Zhi et al. 2012) and protects AMOT from the Nedd4- and Itch-mediated ubiquitination (Wang et al. 2012). Interestingly, all of these reports indicate that TAZ or YAP compete with ubiquitin ligases of the HECT domain family that possess WW domains. The WW domain is a protein interaction domain that facilitates interactions with the amino acid motif: Proline (*P*)—Proline (*P*)—any amino acid (*X*)—Tyrosine (*Y*) motif (also known as a PPXY or PY motif) (Ingham et al. 2004). The WW domains in these ubiquitin ligases share high sequence similarity to the WW domains found in TAZ and YAP. Thus, this shared feature enables TAZ/YAP to compete for target PPXY-protein binding and thereby prevent their ubiquitination.

It has long been known that TGFβ-dependent Smad signaling is restricted by high epithelial cell density (Petridou et al. 2000). Under such conditions, TAZ/YAP are localized in the cytoplasm, and participate in the active cytoplasmic retention of Smads (Varelas et al. 2010b). Interestingly, this role for TAZ/YAP is dominant to TGFβ signals, as Smad complexes are restricted in the cytoplasm by TAZ/YAP even in the presence of high levels of TGFβ ligand. Thus, TAZ/YAP are capable of dictating Smad signal strength and/or defined activity by their localization, an event that will likely prove to be important for propagating TGFβ signals in diseases like cancer. In a tumor-promoting role, TGFβ is capable of driving an epithelial–mesenchymal transition (EMT), which is a process that contributes to the metastatic and stem cell-like properties of aggressive cancers (Thiery et al. 2009). Most epithelial cells do not normally undergo TGFβ-induced EMT, and EMT in tumors is a rarely observable event (Brown et al. 2004). Thus, to achieve EMT-inducing signals TGFβ-induced Smad activity may rely on the presence of high nuclear TAZ/YAP levels. Indeed, driving nuclear TAZ/YAP sensitizes epithelial cells to undergo EMT-like changes (Varelas et al. 2010b), and synergy between TGFβ/Smad signaling and nuclear YAP activity stimulates human malignant mesothelioma growth (Fujii et al. 2012). Interestingly, nuclear TAZ/YAP promote stem cell-like properties in a range of cell

populations (Hiemer and Varelas 2012), including those induced by TGFβ and are found in aggressive breast cancers (Cordenonsi et al. 2011; Mani et al. 2008). Thus, while much remains to be uncovered, the relationship between TAZ/YAP and TGFβ signaling is likely to play a key tumor-initiating role.

Animal development relies on accurate TGFβ signaling, including key roles in the early patterning of the embryo. Deletion of Smad2 together with Smad3 in mice results in a failure of embryos to undergo gastrulation (Dunn et al. 2004). Immediately prior to gastrulation the localization of TAZ/YAP and Smad2/3 is precisely coordinated (Varelas et al. 2010b), suggesting that TAZ/YAP-Smad2/3 signaling is crucial at this stage in development. Cells that make up the visceral endoderm at this stage display hypo-phosphorylated nuclear TAZ/YAP along with nuclear Smad2/3. In the immediately adjacent epiblast region, YAP is hyper-phosphorylated and cytoplasmic. Smad2 is also primarily cytoplasmic and phosphorylated in the epiblast (Varelas et al. 2010b). Smad phosphorylation in the epiblast is indicative of activation by Nodal, a ligand of the TGFβ family that is present in this region of the embryo (Mesnard et al. 2006). Thus, Smads are poised in a phosphorylated and active state at this time prior to gastrulation, competent to initiate transcription. Co-localization with phosphorylated YAP in the epiblast suggests YAP may restrict Smad activity until gastrulation is ready to proceed. Given that Hippo signaling is tightly linked to the mechanosensing properties of cells, the Hippo pathway may have important roles relaying mechanical cues via TAZ/YAP to buffer and/or define TGFβ signals required for gastrulation and other developmental events. Deregulation of these signals will also likely contribute to the onset of disease, but further examination is required to reveal the extent.

17.5.2 Wnt/β-Catenin Signaling

Wnts are secreted factors that signal through Frizzled and LRP cell surface receptors to stimulate a wide range of biological responses (Clevers and Nusse 2012). The transcriptional regulator β-catenin executes many of the Wnt-induced responses in the nucleus, and numerous modes of regulation modulate its nuclear accumulation. Several studies have indicated that Hippo pathway signaling converges with Wnt/β-catenin signaling, and deregulated control of this relationship may contribute to disease. TAZ/YAP are reported to direct β-catenin activity via multiple mechanisms. In the nucleus YAP synergizes with β-catenin to regulate transcription (Heallen et al. 2011). However, in the cytoplasm TAZ/YAP–β-catenin interactions restrict β-catenin from the nucleus (Imajo et al. 2012). TAZ/YAP also bind to the Dvl family of proteins, which are inhibitors of the destruction complex that targets β-catenin for degradation in the absence of Wnt (Varelas et al. 2010a). Cytoplasmic TAZ/YAP binding to Dvl inhibit the phosphorylation and activation of Dvl proteins by Casein Kinase 1δ/ε kinases (Varelas et al. 2010a). TAZ/YAP thereby inhibit Dvl activity in the cytoplasm, enhancing β-catenin turnover and crippling cellular responsiveness to Wnt. Concordantly, TAZ knockdown enhances Wnt-induced Dvl

phosphorylation, stabilizes β-catenin levels, and increases β-catenin-mediated transcription.

Analyses in vivo are consistent with Hippo signaling modulating Wnt/β-catenin activity. For example, cardiac muscle-specific knockout of *Sav*, *Lats2*, or double knockout of *Mst1* and *Mst2* in mice promotes Wnt/β-catenin signaling and leads to enhanced cardiomyocyte proliferation and an enlargement of the heart (Heallen et al. 2011). Knockout of Mst1 and Mst2 from the mouse intestinal epithelium also results in an enhancement of the undifferentiated stem cell population with an increase in β-catenin signaling (Zhou et al. 2011). Additionally, cystic kidneys from *Taz* knockout mice accumulate nuclear β-catenin, consistent with hyperactive Wnt signaling (Varelas et al. 2010a). Analogous regions in wild type kidneys consist of polarized epithelia with cytoplasmic TAZ (Hossain et al. 2007), suggesting that Hippo pathway activity, as inferred from cytoplasmic TAZ, restricts Wnt/β-catenin signals. Taken together, the data suggest that defined Wnt signals are directed by the status of Hippo pathway, and that defects in Hippo signaling translate into uncontrolled Wnt/β-catenin signals to promote the disease state.

17.5.3 Notch

The Notch family consists of conserved single-pass transmembrane receptors that affect cell fate decisions through short-range cell–cell interactions. In mammals several differentially expressed ligands have been identified for the Notch receptors, providing context to the pathway. Recent data has suggested a convergence of Hippo and Notch pathway signals. An association between these pathways was revealed initially from studies examining the epithelium of *D. melanogaster* wing imaginal discs: Hippo pathway mutant epithelial tissue exhibits elevated levels of Notch receptor at the cell surface (Genevet et al. 2009; Maitra et al. 2006). This accumulation was suggested to result from defective membrane trafficking, leading to decreased receptor clearance and a consequential reduction in Notch-dependent transcriptional output (Genevet et al. 2009). Subsequently, mutations in upstream Hippo pathway components were shown to affect Notch signaling in follicle cells of the developing *D. melanogaster* oocyte. Again, mutation of Hippo pathway components led to decreased Notch-mediated transcription, and moreover an increase in the proportion of undifferentiated cells given that Notch promotes differentiation in this context (Polesello and Tapon 2007; Meignin et al. 2007; Yu et al. 2008). Expression of the intracellular domain of Notch (NICD) in these cells was capable of suppressing differentiation phenotypes, suggesting that intact Hippo signaling is normally required to promote Notch signaling in follicle cells.

Like their receptor, *D. melanogaster* Notch ligands Serrate and Delta are altered with respect to levels in tissue lacking functional Hippo pathway components (Cho et al. 2006; Reddy et al. 2010); Serrate levels are increased in mutant tissue of the imaginal disc epithelium (Cho et al. 2006) while optic neuroepithelia from the same mutants display decreased levels of Delta ligand (Reddy et al. 2010).

Thus, a context-dependent relationship between the Hippo and Notch pathways exists. Given the nonautonomous nature of the Notch pathway, Hippo pathway regulation of Notch ligand levels may define distinct niche-dependent events by providing a mechanism to integrate Notch signals with environmental cues.

The conservation of interplay between Notch and Hippo signaling has been suggested from mammalian studies. Conditional expression of YAP in the mouse intestine results in the expansion of undifferentiated progenitor cell populations (Camargo et al. 2007). This expansion can be suppressed with γ-secretase inhibitor treatment, which prevents cleavage and activation of the Notch receptor. Several known Notch target genes are induced upon YAP expression suggesting that YAP promotes Notch activity. Similarly, deletion of Mst1 and Mst2 kinases in the mouse intestine results in activation of Notch-dependent transcription (Zhou et al. 2011), further evidence that nuclear YAP promotes Notch signaling. However, how YAP influences Notch signaling is unclear. Evidence suggests that YAP may influence Notch ligand levels similarly to what has been observed in *D. melanogaster* (Zhou et al. 2011), but clearly dissecting this relationship will be crucial for our understanding of how these pathways converge in development and disease.

17.6 Concluding Remarks

Work from many laboratories has revealed complex and extensive roles for Hippo pathway components that touch on many unexpected functions critical for animal development. Such functions include defining epithelial apical–basal and PCP, controlling aspects of ciliogenesis, and directing the output of other developmentally important signaling pathways. Mechanistically, influence on these processes by Hippo pathway components go beyond direct transcriptional control and include roles in guiding protein complex formation, protein stability, and protein localization. Diverse mechanisms regulate Hippo pathway activity, many of which provide context and specificity to the wide-ranging functions of the pathway. The complexity of regulation by and of Hippo signaling is illustrated by direct links with architectural and mechanical intracellular cues as well as cross talk with other signaling pathways. Thus, understanding the mechanisms directing the localization of Hippo pathway effectors and dissecting the mechanisms interconnecting the Hippo pathway with other pathways is of clear importance.

The Hippo pathway has essential roles in development and disease, and it is almost certain that many aspects of Hippo signaling are undiscovered. With the field of researchers studying the Hippo pathway increasing, it is likely that more unexpected roles will be revealed that will take the pathway beyond its traditionally considered functions. Understanding how these canonical and non-canonical signals are interconnected is a critical next step needed for manipulating pathway activity, and mechanistic clarity is required to aid in the effective design of therapeutics that can target the range of diseases that accompany Hippo pathway deregulation.

Acknowledgements Work in XV's laboratory is supported in part by Research Grant No. 5-FY11-578 from the March of Dimes Foundation and by funds from the Concern Cancer Foundation.

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M. Oren and Y. Aylon (eds.), *The Hippo Signaling Pathway and Cancer*,
DOI 10.1007/978-1-4614-6220-0, © Springer Science+Business Media New York 2013