Drosophila Eye as a Model to Study Regulation of Growth Control: The Discovery of Size Control Pathways

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Introduction

In the biological sense, the term growth has intricate ramifications that we have only started to comprehend. Growth is the overall increase in cell mass or size of a tissue or organism (Conlon and Raff 1999; Cook and Tyers 2007; Edgar 1999; Raff 1996). Growth may be due to increase in cell number resulting from cell division (cell proliferation), increase in cellular mass without cell division (cell enlargement), or due to release of more extracellular matrix (cell accretion). These processes are intimately linked and it is clear that if coordinated growth has to occur in an organism, it is necessary for various biological pathways to interact and relay appropriate signals to proper cell types. Growth regulation is precisely controlled and affected by several intrinsic and extrinsic factors (Cooper 2004; Crickmore and Mann 2008; Grebien et al. 2005; Johnston and Gallant 2002). The intrinsic factors mainly involve synthesis and secretion of signals or ligands, which bind to their cognate receptors to relay downstream signals. These signals consist of variety of molecules such as hormones, mitogens, apoptosis-inducing signals, patterning and axis determining signals, etc.

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which eventually determine organ size and tissue homeostasis (Johnston and Gallant 2002; Mitchison et al. 1997; Montagne 2000; Tumaneng et al. 2012a). Growth of a tissue or organ is impacted not only by cell division but also by regulated cell death (apoptosis or programmed cell death; Bangs and White 2000; Jacobson et al. 1997; Martin et al. 2009; Oldham et al. 2000a; Richardson and Kumar 2002; Rusconi et al. 2000).

In this chapter, we will focus on growth regulation in imaginal discs (epithelial sacs that are precursors of adult appendages) in *D. melanogaster* (Bergantinos et al. 2010; Bryant 1978; Bryant 1987; Bryant 2001; Bryant and Schmidt 1990). The obvious advantages that *Drosophila* has to offer as a model organism include short life cycle, high fecundity, low cost maintenance, and lack of redundancy in genome (Bier 2005; Blair 2003; Boutros and Ahringer 2008; Pagliarini et al. 2003; St. Johnston 2002; Vidal and Cagan 2006). Furthermore, the sophisticated tools available in fly genetics provide great deal of versatility in terms of designing experiments. The plethora of knowledge thus generated through exhausting efforts of scientists has not only revealed to us the classic information about how growth occurs but has also lead to better understanding of growth-related diseases such as cancer.

Drosophila Eye as a Model to Study Regulation of Growth

The compound eyes of *Drosophila* arise from the eye-antennal imaginal discs, a monolayer epithelial sheet of cells that is responsible for the development of the eyes, the antennae, the ocelli, and a major part of the adult head cuticle. Each eye of the adult fruit fly on an average consists of about 800 ommatidia (Wolff and Ready 1993). Ommatidia arise from a set of 19 precursor cells that are generated by spatially and temporally coordinated cellular processes such as cell-proliferation, cell-differentiation, and cell-death in the eye imaginal discs. Eighteen of these cells contribute to the eye per se, whereas the nineteenth cell gives rise to a sensory bristle (Cagan 1993). A key feature that distinguishes eye from the rest of the organs is its ability to perceive light and relay the signal to distinct areas in the brain called the optic lobes. The eye imaginal discs arise from about 50 primordial cells that express the *Drosophila* PAX 6 gene *eyeless* (*ey*) during mid-to-late embryogenesis. Two such discs develop in each larva and differentiate into two compound eyes, antennae, ocelli, and the head cuticle in the adult.

Much is known about the regulation of growth and differentiation of the eye-antennal imaginal discs (Baker 2001; Cagan 1993; Dominguez and Casares 2005; Hafen 1991; Kramer and Cagan 1994; Kumar 2001). Until the second larval instar of development, the cells of the eye-antennal discs proliferate without differentiation (Baker 2001; Wolff and Ready 1993). During the second instar stage, a unique process of cell differentiation begins in the eye-antennal disc that paves the way for formation of photoreceptor neurons in the posterior region of the eye-antennal imaginal disc (Wolff and Ready 1993). The differentiation occurs in the wake of a so-called "morphogenetic furrow"—a front marked by apical constriction of epithelial cells in response to complex developmental signaling from the Hedgehog (Hh), Decapentaplegic (Dpp), Wingless (Wg), and Epidermal growth factor receptor (EGFR) pathways (Acquisti et al. 2009; Chen and Chien 1999; Firth et al. 2010; Harvey et al. 2001; Kango-Singh et al. 2003; Penton et al. 1997). Posterior to the morphogenetic furrow, the cells begin to acquire particular photoreceptor cell fates and organize into ommatidial clusters.

Anterior to the furrow, the cells divide asynchronously and do not differentiate, however, in the morphogenetic furrows, cells arrest in the G1 phase of the cell cycle, synchronize, and either start to differentiate into photoreceptor cells as they leave the furrow or undergo one additional round of cell division, referred to as the second mitotic wave (SMW) before differentiating into the remaining photoreceptor, cone, pigment, and bristle cells (Baker 2001; Dickson and Hafen 1993; Wolff and Ready 1993). The cells posterior to the morphogenetic furrow enter G1 arrest caused by Dpp (*decapentaplegic*) signaling that is maintained by the *roughex* (*rux*) gene, which negatively regulates G1-S transition. The cells that are temporarily trapped in the G1 phase begin differentiation with specification of the R8 (photoreceptor) cell due to expression of the proneural protein Atonal (Ato) (Baker et al. 1996; Chen and Chien 1999; Daniel et al. 1999; Dominguez 1999; Greenwood and Struhl 1999; Jarman et al. 1994). R8 recruits other photoreceptor cells-R2, R3, R4, and R5 to form a cluster of five photoreceptor precursors. Once specified, these cells never enter cell cycle or cell division again. All other nonspecified cells re-enter cell cycle only once at the SMW (Baker 2001; de Nooij and Hariharan 1995). Cells in the SMW undergo G2/M phase that is mediated through local signaling from Spitz (Spi). Binding of Spi to its cognate receptor EGFR in precursor cells causes activation of downstream *string* (*stg*) that completes the G2-M transition during mitosis. Local Spi-EGFR signaling also plays an important role limiting the progression of SMW. For instance, on an average the Spi signal from one precluster can span to a length of seven cells only causing these cells to divide whereas the remaining cells remain arrested in G2 phase and fail to divide (Baker 2001; Brumby and Richardson 2003; de Nooij and Hariharan 1995; Jarman et al. 1994; Price et al. 2002; Wolff and Ready 1991). The progression of the morphogenetic furrow is complete by the mid-third instar of larval development, and the eye-antennal disc is fully grown to about 50,000 cells (Kumar 2009; Kumar and Moses 2000; Kumar and Moses 2001; Sun 2007).

Following development in larval stages, supernumerary cells are eliminated via apoptosis during pupal development. This event is mediated through Notch signaling (Bonini and Fortini 1999; Burke and Basler 1997; Sawamoto and Okano 1996; Treisman and Heberlein 1998; Zipursky 1989). By contrast, survival of pupal cells is brought about by EGFR expression that mediates its cell survival function through suppressing the transcriptional activity of the proapoptotic gene *head involution defective* (*hid*) (Bonini and Fortini 1999). In addition, survival signals emanating from cone or primary pigment cells in each ommatidium play a role in survival and proliferation of secondary and tertiary pigment cells, and secondary bristle organs (Cagan 1993, 2009; Rubin 1989; Singh et al. 2012; Tsachaki and Sprecher 2012; Yamamoto 1993). During metamorphosis, the two eye-antennal imaginal discs fuse at the dorsal midline to form the fly head with three ocelli, two antennae, and compound eyes.

Thus, the eye-antennal disc is ideal for the study of organogenesis, morphogenesis, pattern formation, and several cell biological processes including the regulation of cell cycle, cell death, cell junctions and adhesion, transport of molecules, cell signaling, and metabolism. Recently, the eye discs have been used as an experimental system for genetic screens to discover postembryonic lethality, and for screening small molecule inhibitors in chemical and drug screens.

The Mosaic Analysis Systems and the *Drosophila* Eye

Mutagenesis screens are a very well-established tool for gene discovery in flies (for review, see Bellen et al. 1989, 2011; Blair 2003; Pfeiffer et al. 2010; St. Johnston 2002; Venken and Bellen 2012; Xu and Rubin 1993). Over the years, the *mosaic techniques* have evolved to include the Flipase(*FLP*)-Flipase recognition target (*FRT*), *eyGAL4 UASFlp EGUF*, Flp-out clones, and Mosaic Analysis with Repressible Cell Marker MARCM (for review, see Blair 2003; St. Johnston 2002). One of the first tissue-specific mosaic systems was developed in the eye-antennal discs where the mosaic clones were restricted to the eye-antennal discs by virtue of expression of the Flippase gene under the control of the Eyeless Promoter (commonly referred to as the '*ey-FLP* system', Newsome et al. 2000). This tissue-specific system was further refined by the development of the "*cell-lethal*" system, where effects of loss of function of a gene could be surveyed more clearly because the wild-type twin-clones are eliminated due to the presence of *cell-lethal* mutations (the *cell-lethal FLP-FRT* system; Newsome et al. 2000). We focus on the genetic screens performed about 10–12 years ago (simultaneously in many labs) that led to the identification of many new genes that were shown to belong to the two major growth regulatory networks: the Hippo pathway and the Tuberous Sclerosis Complex/Target of Rapamycin—TSC-TOR pathway.

Genetic Screens for Genes That Regulate Growth: The "Big-Head" and "Pin-Head" Mutations

Barry Dickson's group (Newsome et al. 2000) improved the traditional FLP-*FRT* approach developed in the Rubin Lab (Xu and Rubin 1993), to allow generation of essentially mutant eye discs by eliminating the wild-type twin clone via a *cell-lethal* mutation (the *cell-lethal* FLP-*FRT* system) (Fig. 1a). This so-called "*cell-lethal*" approach allows the mutant clones to grow to their highest potential due to elimination of competitive interactions between the mutant cells and their wild-type neighbors. Using this system, several groups carried out mutagenesis screens in flies (on the X, 2L, 2R, 3L, 3R chromosomes) and found mutations that affected patterning, growth, cell death, and differentiation (for review, see St. Johnston 2002).

Of special interest were the genes mutations which caused a remarkable effect on growth without disrupting the patterning process (Conlon and Raff 1999; Johnston and Gallant 2002; Mitchison et al. 1997; Oldham et al. 2000a; Raff 1996; Su and

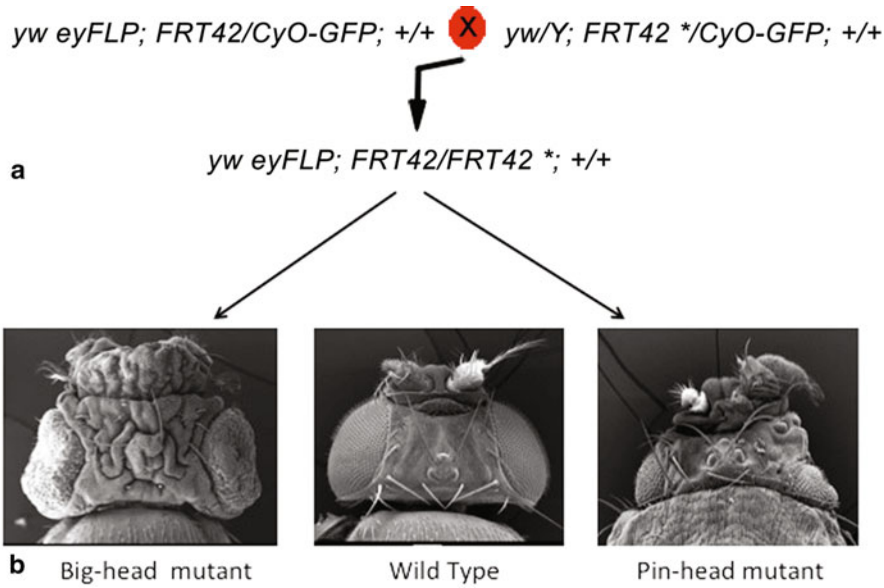

Fig. 1 Genetic screens for eye-specific mosaics lead to the identification of several Hippo and TSC-TOR pathway mutants. **a** Modified mutagenesis scheme where * represents a point mutation induced by a mutagen (e.g., Ethyl Methane Sulphonate (EMS)), **b** Typical phenotypes of Hippo and TSC-TOR pathway mutant from the mutagenesis screen

O'Farrell 1998; Tumaneng et al. 2012a). Characterization of these mutants revealed the mechanisms that regulate growth and tissue size by controlling cell number (Hippo pathway) (Zhao et al. 2011b) or cell size (InR/TSC-TOR pathway) (Kim and Guan 2011; Loewith 2011; Montagne 2000; Potter et al. 2003; Soulard et al. 2009) in a developing organ. Typically, loss of function mutations in positive regulators of these pathways caused development of enlarged heads that showed overgrowth—referred to as the "big head" mutations (Fig. 1b) (Hafen 2004; Oldham and Hafen 2003; Pan 2007, 2010). In contrast, loss of function of negative regulators of these pathways caused reduction in head size and development of smaller organs, which may be due to cell death or reduction in cell size, and were referred to as the "pin head" mutations (Fig. 1b).

The Hippo Signaling Pathway

The Hippo signaling pathway was first discovered in flies following characterization of "big-head" mutants identified from genetic screens (for review, see Edgar 2006; Pan 2007; Saucedo and Edgar 2007). Analysis of the loss of function phenotypes revealed that a fundamental function of the Hippo pathway was the regulation of organ size (Boggiano and Fehon 2012; Harvey and Hariharan 2012; Schroeder and Halder 2012; Staley and Irvine 2012). Interestingly, the pathway received its name just after some growth regulatory genes (*warts* (*wts*), *salvador* (*sav*, aka *shar-pie, shrp*)) were characterized. Warts (*wts*) was named based on the bumpy "warts-like" phenotype of the mutant cells in mitotic (mosaic) clones on the body of the adult flies that were reminiscent of the warts on toads (Justice et al. 1995). Another group led by Xu et al. (1995) also independently found *warts* in the initial FLP/*FRT*-based screen and named it *large tumor suppressor* (*LATS*) (Xu et al. 1995). Two independent groups identified the gene encoding the adaptor protein Salvador (Sav aka Shar-pie, Shrp after the dog species of the same name as the mutant flies showed a characteristic phenotype of folded dark cuticle on the overgrown heads) from complementation groups isolated from the big-head genetic screens (Kango-Singh et al. 2002; Tapon et al. 2002). Interestingly, both Wts and Sav regulated growth by suppressing proliferation and promoting apoptosis. Hippo (Hpo) was the name given to another complementation group from the "big-head" screens that showed a phenotype that was very similar to Wts and Sav mutants (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003).

Molecular analysis of the three genes revealed that Wts and Hpo genes encode for serine-threonine (S-T) kinases whereas Sav is a WW domain containing adaptor protein. By this time it was clear that Warts, Salvador, and Hippo all show similar loss of function phenotypes and control organ size by a common signaling pathway that promotes apoptosis and restricts cell proliferation (Edgar 2006; O'Neill and Kolch 2005; Rothenberg and Jan 2002), and the pathway got its name from the last member of this trio of genes. A complete pathway that relays a growth regulatory signal from the plasma membrane to the nucleus has emerged over the last decade. Although genetic mutagenesis screens led to the initial discovery of this pathway, several components were identified by other genetic screening strategies and biochemical approaches (e.g., yeast-two hybrid screens, TAP-TAG based protein interaction assays; for review, see Halder and Johnson 2011; Kango-Singh and Singh 2009; Staley and Irvine 2012; Tumaneng et al. 2012a; Varelas and Wrana 2012). Today the Hippo pathway has grown to a large network of tumor suppressor genes that function upstream and downstream of the three initial members of the Hippo pathway (also known as the core kinase cascade) that control several aspects of tissue homeostasis. Overall, the Hippo signalling pathway is a key size regulatory pathway that controls organ size in flies and vertebrates, and misregulation of Hippo signalling is implicated in several diseases including cancer (for review, see Harvey and Hariharan 2012; Schroeder and Halder 2012; Staley and Irvine 2012; Zhao et al. 2011b; Fig. 2).

Regulation by Core Kinase Cascade of the Hippo Pathway

The molecular analysis of the three initial members of the Hippo pathway in *Drosophila* revealed that Hpo codes for an S-T kinase of the mammalian Sterile-20 family of kinases (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003), and can physically associate with the WW-domain containing adaptor protein Sav (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003;

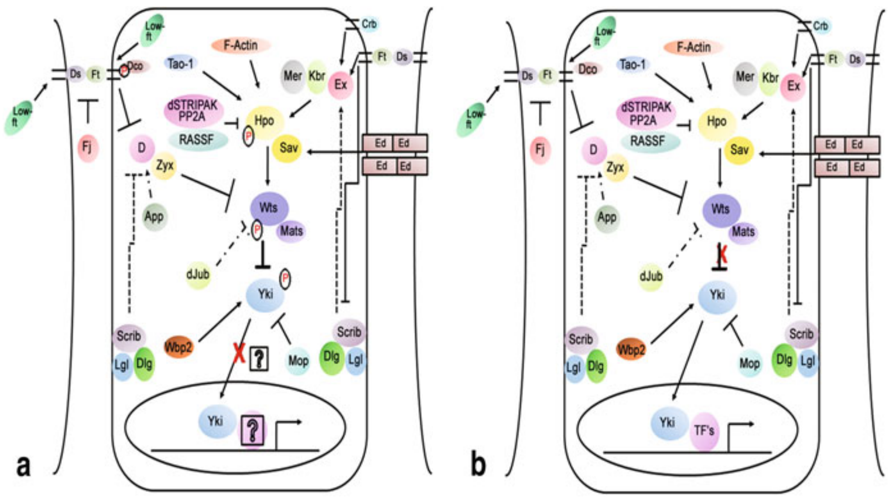

Fig. 2 Schematic representation of the Hippo pathway in *D. melanogaster*. **a** Signaling interactions when Hippo pathway is downregulated in response to extracellular signals. Hpo fails to phosphorylate Wts. Inactive Wts cannot phosphorylate Yki, and allows Yki to enter the nucleus to bind cognate transcription factors and induce expression of target genes. **b** Signaling interactions when Hippo pathway is activated by stress. Hpo is phosphorylated and in turn phosphorylates Wts with the help of adaptor proteins Sav and Mats. Activated Wts phosphorylates Yki and prevents it from entering the nucleus, thus preventing transcription of target genes. In addition, cell death is induced when the pathway is hyperactivated

Udan et al. 2003; Wu et al. 2003). Wts is an S-T kinase protein of the dystrophia myotonica protein kinase (DMPK) family that associates with another adaptor protein Mob as tumor suppressor (Mats; Justice et al. 1995; Lai et al. 2005; Shimizu et al. 2008; Wei et al. 2007; Xu et al. 1995). Loss of function of these genes in genetic mosaics revealed strong overgrowth phenotype caused by increased cell proliferation and diminished sensitivity to apoptosis. Hyperactivation of the pathway by over-expression of Hpo, Sav, Wts, or Mats leads to formation of smaller organs due to increased apoptosis (Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wei et al. 2007; Wu et al. 2003). Biochemical analysis showed that the Hpo kinase phosphorylates and can physically associate with Sav, Wts, and Mats to form protein complexes in vitro (Wei et al. 2007). However, Hpo associates with its cognate adaptor protein Sav to form the Hpo-Sav complex for efficient activation of the downstream kinase Wts (Huang et al. 2005; Wu et al. 2003). Wts itself associates with Mats to form the downstream Wts-Mats complex of the core kinase cascade of the Hippo pathway (Wei et al. 2007). Association of these adaptor proteins is known to stimulate the catalytic activity of the Hpo and Wts kinases (Dong et al. 2007; Pan 2007; Wei et al. 2007). Moreover, phosphorylation of Mats by the Hpo kinase increases its affinity for the Wts kinase (Dong et al. 2007; Pan 2007; Pan 2010; Wei et al. 2007). Wts is activated by autophosphorylation and phosphorylation by Hpo-kinase. Activated Wts associates with Mats (thus Mats cannot simultaneously associate with Hpo and Wts), which acts as a coactivator for the kinase activity of

Wts (Dong et al. 2007; Huang et al. 2005; Oh and Irvine 2008; Oh and Irvine 2009). A major output of the core kinase cascade is to inhibit the growth-promoting activity of Yorkie (Yki), the *Drosophila* homolog of the mammalian Yes-associated protein (YAP) oncogene that acts as a transcriptional coactivator (Dong et al. 2007; Huang et al. 2005). Yki was identified via a yeast two-hybrid screen as an interactor of Warts. Over-expression of Yki phenocopies the loss of function of *hpo, sav, wts,* and *mats* (all genes of the core kinase cascade) and causes over-growth (Dong et al. 2007; Wei et al. 2007). Loss of function of *yki* results in formation of smaller organs due to induction of cell death (Huang et al. 2005).

Yki activity is regulated by controlling its subcellular localization via phosphorylation-dependent and -independent interactions with the core kinase cascade of the Hippo pathway (Oh and Irvine 2008, 2010; Ren et al. 2010b). Yki associates with Wts, and one mechanism by which the Wts-kinase restricts Yki activity is via phosphorylation at Ser168 that creates a 14-3-3 protein-binding site (Goulev et al. 2008; Peng et al. 2009; Ren et al. 2010b; Wu et al. 2008; Zhang et al. 2008b; Zhao et al. 2008b). Interestingly, only phosphorylated forms of Yki can associate with 14-3-3 proteins. Yki is phosphorylated at multiple sites (e.g., Ser 111 and S250), making it less sensitive to Hpo/Wts-mediated inhibition. These phosphorylation events act in parallel to phosphoYki/14-3-3 mediated mechanisms and inhibit Yki nuclear localization and activity. It is suggested that nuclear export is required for shuttling Yki to the nucleus in response to Hpo signaling, and binding of 14-3-3 proteins is thought to impede nuclear import and/or promote nuclear export thereby facilitating nucleocytoplasmic shuttling of target proteins (Brunet et al. 2002; Kumagai and Dunphy 1999). Nuclear transport of Yki depends on its binding with cognate transcription factors as Yki does not have an intrinsic nuclear localization signal (NLS) (Goulev et al. 2008; Zhang et al. 2008b). Currently, it is unclear if binding of 14-3-3 proteins to Yki prevents its binding with cognate transcription factors, or masks the NLSs or promotes export from the nucleus. Nevertheless, coactivator Yki/YAP is the critical downstream regulatory target of the Hpo kinase cascade, and regulation of its subcellular localization is the primary mechanism by which the Hpo pathway influences target gene expression (Goulev et al. 2008; Huang et al. 2005; Oh and Irvine 2008, 2009, 2010; Oh et al. 2009; Peng et al. 2009; Ren et al. 2010b).

Yki (like Sav) is a WW-domain-containing protein and interacts with the PPxY (where P = Proline; x = any amino acid; Y = Tyrosine) motifs in Wts (Huang et al. 2005). Besides Wts, the WW-domains of Yki interact with the PPxY motifs present in other components of Hippo signaling pathway like Expanded (Ex), Hpo, WW-domain-binding protein 2, and Myopic to regulate Hippo signaling via phosphorylation-independent mechanisms (Badouel et al. 2009; Gilbert et al. 2011; Oh et al. 2009; Zhang et al. 2011b). Another protein that acts via its WW-domains is Kibra which associates with the PPxY motifs in Ex (and binds Mer in a WW-domain independent manner; Baumgartner et al. 2010; Genevet et al. 2010). The identification of multiple proteins that act through the interaction between WW-domains and PPxY motifs in the Hippo pathway suggests that these motif-specific interactions are important for regulation of Hippo signaling (reviewed in Sudol (2010); Sudol and Harvey (2010)).

Yki Activity and Regulation of Expression of Target Genes

Hyperactivation of the pathway, for example, by overexpression of Hpo, leads to phosphorylation and activation of Hpo and Wts with the help of adaptor proteins Sav and Mats. Wts, in turn, phosphorylates the transcriptional coactivator Yki, which associates with 14-3-3 proteins and remains sequestered in the cytoplasm (Dong et al. 2007; Huang et al. 2005; Oh and Irvine 2008; Oh et al. 2009; Ren et al. 2010b). Analysis of adult and imaginal disc phenotypes reveals that over-expression of Hpo results in induction of ectopic apoptosis early in development in imaginal disc cells due to induction of caspase-dependent cell death (Hamaratoglu et al. 2006; Harvey et al. 2003; Udan et al. 2003; Verghese et al. 2012a). In mammalian cells, activation of MST (Mammalian Sterile-20 like kinase)1/2 and hyperphosphorylation of YAP2 by MST2 and LATS1 kinase leads to activation of cell death. Interestingly, MST1/2 are known targets of caspases and YAP1/2 are known to interact with p73 via a PDZ domain in YAP, and induce apoptotic target genes (Bertini et al. 2009; Sudol 2010; Sudol and Harvey 2010). However, these mechanisms of regulating apoptosis may not be conserved in flies because the site for caspase cleavage is not conserved in *Drosophila* Hpo (Wu et al. 2003), and *Drosophila* Yki does not have the conserved PDZ domain (Sudol and Harvey 2010). Nevertheless, Hpo overexpression in flies induces apoptosis through an alternate mechanism that does not involve caspase cleavage or p73. Recently, it was shown that the effector caspase Dronc (*Drosophila* homolog of mammalian Caspase 9) is induced in conditions when Hippo pathway is hyperactivated. Further, using reporter genes, it was shown that *dronc* transcription is induced during gain-of-function and downregulated during loss-of-function conditions of the Hippo pathway, suggesting that *dronc* is a transcriptional target of the Hippo pathway (Verghese et al. 2012a). However, the molecular mechanism by which Yki interacts with Dronc remains unclear. Both phosphorylation-dependent (e.g., with 14-3-3 by phosphorylation-dependent mechanisms) and phosphorylation-independent mechanisms (binding with Hpo, Wts, or Ex) result in cytoplasmic retention of Yki in multiple protein complexes. Thus, the possibility remains that hyperactivation of Hippo pathway, releases Yki from one or more cytoplasmic complexes to allow its binding to transcription factors and shuttle into the nucleus to induce *dronc* transcription. Alternatively, hyperactivation of the Hippo pathway involves a transcriptional repressor that acts together with or independent of Yki to control *dronc* expression. Thus, although it is clear that hyperactivation of the Hippo pathway leads to induction of apoptosis, the molecular mechanisms underlying this process are yet unidentified.

When the pathway is downregulated, the genes of the core kinase cascade act as tumor suppressors by suppressing the growth-promoting activity of Yki. Under these conditions, Yorkie can partner with transcription factors like the TEAD family protein, Scalloped (Sd) and enter the nucleus and cause transcription of target genes which regulate cell proliferation and apoptosis. Sd was identified as the transcriptional factor of the pathway via yeast two-hybrid screen, and in-vitro Yki activity assays (luciferase assay) (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008b). Sd is required for wing development (Campbell et al. 1992;

Liu et al. 2000), whereas Yki is required for regulating growth of all imaginal disc cells. Other transcription factors that bind Yki to regulate growth via Hippo signaling have since been discovered. These include Mothers Against Dpp (Mad) (Alarcon et al. 2009; Oh and Irvine 2010; Peng et al. 2009), Homothorax (Hth), and Teashirt (Tsh) (Peng et al. 2009). Mad is a known transcription factor within the Dpp/tumor growth factor (TGFβ) signaling pathway, and Mad and Hth were shown to control the activity of the *bantam miRNA* (Alarcon et al. 2009; Peng et al. 2009). Mad, Hth, and Tsh are known transcription factors that respond to other signals and are required for patterning of imaginal discs during development.

Yki activity is controlled by the upstream signals (Grusche et al. 2010; Oh and Irvine, 2010). A large number of target genes have been identified over the past decade, which include the cell cycle regulators E2F1, and *cyclin E, A, B, D*; the growth promoter *Myc,* and cell survival-promoting miRNA *bantam*, genes regulating cell death like the *Drosophila inhibitor of apoptosis diap1*, *hid, dronc*; and cytoskeletal proteins like *f-actin*, which drive cell proliferation and cell survival (Fig. 3)(Goulev et al. 2008; Harvey et al. 2003; Huang et al. 2005; Jia et al. 2003; Kango-Singh et al. 2002; Neto-Silva et al. 2010; Nolo et al. 2006; Pantalacci et al. 2003; Peng et al. 2009; Tapon et al. 2002; Thompson and Cohen 2006; Udan et al. 2003; Wu et al. 2003; Wu et al. 2008; Zhang et al. 2008a; Ziosi et al. 2010). Yki also controls the expression of several upstream components of the Hpo pathway like Ex, Mer, Kibra, Crumbs (Crb) and Four-jointed (Fjose et al. 1984) by a negative feedback loop (Cho et al. 2006; Fjose et al. 1984; Genevet et al. 2009, 2010; Hamaratoglu et al. 2006). Recently, Yki was shown to affect the expression of components of other signaling pathways, such as ligands for the Notch, Wnt, EGFR, and Jak-Stat pathways (Cho et al. 2006; Karpowicz et al. 2010; Ren et al. 2010a; Shaw et al. 2010; Staley and Irvine 2010, 2012; Zhang et al. 2009a). These interactions suggest that Hippo pathway interacts with the major signal transduction pathways, and these points of contact between different pathways may play an important role in controlling correct tissue sizes and maintaining homeostasis (Fig. 4).

Genetic and biochemical studies thus provide a basic premise for how Yki activity is modulated when Hippo signaling is downregulated or upregulated (Halder and Johnson 2011; Harvey and Hariharan 2012; Schroeder and Halder 2012; Staley and Irvine 2012). Studies in imaginal discs and other cell types like intestinal stem cells and fat cells revealed that Hippo signaling is needed in all cell types to regulate growth, and that the activity of the pathway is modulated to achieve tissue homeostasis (Halder et al. 2012; Halder and Johnson 2011; Harvey and Hariharan 2012; Tumaneng et al. 2012a; Zhao et al. 2008a; Zhao et al. 2010a). Whether Hippo signaling pathway is regulated by other global instructive signals (e.g., morphogen gradients) or if the pathway is constitutively active remains unknown. However, several inputs that communicate a growth regulatory signal to the core kinase cascade have been identified. We will discuss the key inputs, and their connection to the core kinase cascade in the following sections.

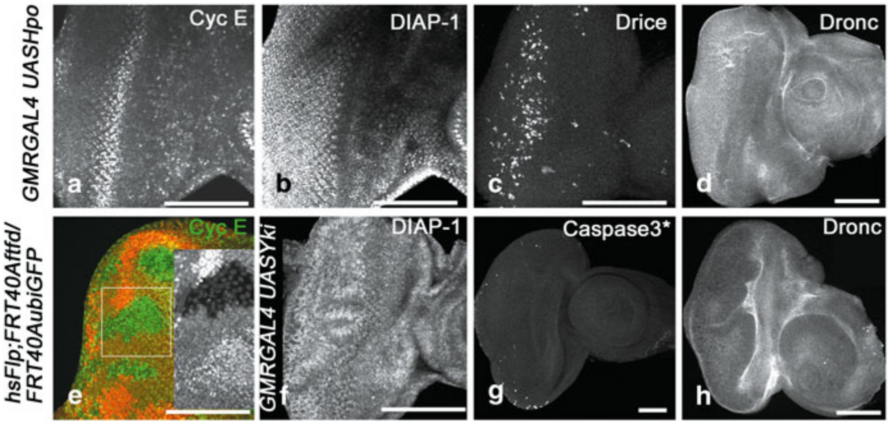

Fig. 3 Hippo pathway target genes regulate cell proliferation and apoptosis. (**a–d**) *GMRGAL4 UASHpo* third instar eye-antennal imaginal disc showing affect on target proteins upon pathway hyperactivation in the GMR domain. **a** Cyc E is downregulated, **b** DIAP-1 levels remain unaffected, and **c** Drice is activated (Drice is the homolog of *Drosophila* Caspase3* and is a read-out of active Dronc), **d** Dronc is upregulated in the GMR domain upon Hpo overexpression. **e** Loss of function clones of *ft* (GFP negative) made with yw hsFLP; UbiGFP [*hsFLP; FRT40A* ft^{fd}*/FRT40A ubiGFP*] show upregulation of Cyc E in the mutant cells. This effect is very strong in the region of the SMW. (**f–h**) *GMRGAL4 UASYki* third instar eye-antennal imaginal discs. **f** DIAP-1 is up-regulated, **g** Caspase3* staining is not observed, and **h** Dronc is down-regulated in the GMR domain consistent with overproliferation and no apoptosis

Upstream Regulators of the Hippo Pathway

Since the discovery of the core kinase cascade, several upstream regulators of the Hippo pathway were identified. These discoveries highlighted two remarkable properties of the Hippo pathway—one, that the Hippo pathway is a signaling network with multiple points of signal integration rather than a linear system of epistatic genes; and two, the interactions between various protein complexes (at the signal integration points) may play a decisive role in shaping the outcome, i.e., Yki activity levels. Although our understanding of the network is incomplete in both these areas, it is clear that signaling interactions within this pathway are shaped by several distinct inputs.

I. Fat signaling and the Hippo Pathway *Fat* (*ft*) alleles were spontaneous mutations first described by Mohr (1923, 1929). Subsequent analysis of mutations in the *ft* locus revealed both viable and lethal alleles, of which the null alleles are larval lethal and show hyperplastic overgrowth of imaginal discs thereby acting as tumor suppressor genes (Bryant et al. 1988). Molecular cloning of *ft* revealed that it codes for a transmembrane protein, which is an atypical Cadherin (Mahoney et al. 1991). Loss of *ft* affects two distinct aspects of imaginal disc growth and development, restriction of cell proliferation and generation of correctly oriented cells within the epithelial sheet, phenotypes that were mapped to two distinct signaling pathways—the Hippo and the Planar Cell Polarity (PCP) pathway (see (Brittle et al. 2010; Cho et al. 2006; Matakatsu and Blair 2006; Matakatsu and Blair 2008; Matakatsu and Blair 2012)). Ft is ubiquitously expressed, however its functions are regulated by two genes, Dachsous (Ds) and Fj, which are expressed in gradients in developing tissues (Matakatsu and Blair 2004; Reddy and Irvine 2008). Ds is another proto-cadherin in flies that acts as the ligand for Ft for both the Hippo and PCP pathways (reviewed in (Thomas and Strutt 2012)). Fj is a Golgi-localized kinase that phosphorylates the extracellular Cadherin domains of Ft and Ds to promote their binding (Ishikawa et al. 2008; Simon et al. 2010). Phosphorylation of Fat by Fj increases its affinity to Ds while phosphorylation of Ds reduces its affinity to Ft. One way in which Fat regulates growth and PCP is based on the slope and vector of the Ds and Fj gradients (Halder and Johnson 2011; Willecke et al. 2008; Zecca and Struhl 2010) .

Several years after Ft was discovered, it was realized that the growth regulatory functions of Fat were tied to the Hippo pathway (Bennett and Harvey 2006; Cho et al. 2006; Silva et al. 2006; Willecke et al. 2006). Loss of *ft* in mutant clones phenocopied the loss of function phenotypes of genes within the core kinase cascade of the Hippo pathway. Imaginal discs containing somatic clones of *ft* mutant cells continued to proliferate when normal cells had stopped, thereby forming large overgrown discs. Transcriptional targets of Hippo pathway are induced within the *ft* mutant cells, a phenotype similar to loss of function of positive regulators of Hippo pathway (e.g., *wts, hpo, sav, mats*). Ft affects the levels and localization of Hippo pathway components, including Wts, Ex, and Yki (Bennett and Harvey 2006; Cho et al. 2006; Oh and Irvine 2008; Silva et al. 2006; Tyler and Baker 2007; Willecke et al. 2006). Ft influences Hippo signaling independent of other upstream regulators like *expanded, merlin* (*mer*), and *kibra* which form a heteromeric complex (Ex-Mer-Kibra), and other genes like the Tao-1 kinase (Boggiano et al. 2011; Poon et al. 2011) that act upstream of Hpo (Boggiano and Fehon 2012). However, several other genes were recently identified, which specifically act downstream of Ft and integrate with the Hippo pathway by influencing the activity of the downstream kinase Wts. Thus, the Fat branch of the Hippo pathway has emerged, which independently influences Wts activity and tissue growth (Halder and Johnson 2011; Kango-Singh and Singh 2009; Reddy and Irvine 2008; Staley and Irvine 2012).

Several components of the Ft branch influence the intracellular domain of Ft—the region critical for transducing the signal within cells. These include the *Drosophila* Discs overgrown (Dco), a homolog of Casein Kinase I, that phosphorylates the Ft intracellular cytoplasmic domain in a Ds-dependent manner (Cho et al. 2006; Feng and Irvine 2009; Sopko et al. 2009); and the unconventional myosin Dachs (D) (Cho et al. 2006; Cho and Irvine 2004; Mao et al. 2006). Loss of function of dco^3, a hypomorphic allele, in homozygous discs and in somatic clones result in tissue overgrowth, and shows elevated levels of Fj and Diap-1 (Bryant and Schmidt 1990; Feng and Irvine 2009; Guan et al. 2007). Dco binds to the cytoplasmic domain of Fat, and in *dco* mutants, Fat intracellular domains fail to phosphorylate. Ds enriches availability of Fat at the point of cell contacts by forming *cis*-dimers with Fat. This promotes the transphosphorylation of Fat by Dco. Lowfat is a novel protein that interacts with the intracellular domains of Fat and Ds, and stabilizes the Fat-Ds interaction (Mao et al. 2009). Lowfat was identified in a genome-wide yeast two-hybrid screen as a Fat- and Ds-interacting protein (Mao et al. 2006, 2009). In addition, the palmitoyltraserase Approximated (App) acts downstream of Ft, and Ft regulates the localization of D to the membrane through APP (Matakatsu and Blair 2008). Recently, the apical-basal polarity gene *scribble* (*scrib*) (Verghese et al. 2012b) and the LIM (Lin-1; Isl-1; Mec-3)-domain protein *zyxin 102* (*zyx*) (Rauskolb et al. 2011) were shown to act in the Fat branch of Hippo signaling pathway (Bennett and Harvey 2006; Cho et al. 2006; Meignin et al. 2007; Polesello and Tapon 2007; Reddy et al. 2010; Silva et al. 2006; Willecke et al. 2006).

The differences in Ds and Fj expression between neighboring cells stimulate Yki activity, whereas the vector property of the gradients effects PCP signaling. Localization of D to the membrane is regulated by Fj, Ds, and Ft (Cho et al. 2006; Mao et al. 2006; Rogulja et al. 2008; Willecke et al. 2008). D controls Yki activity by two alternative mechanisms, one, involves post-translational effects of Ft on Wts, and the second involves the localization of Ex to the subapical membrane (Bennett and Harvey 2006). The apical basal polarity gene *scrib* and the atypical myosin D are responsible for partitioning the growth regulatory signal from Ft to downstream genes. Genetic epistasis experiments placed Ft upstream of D, and the apical regulator of the pathway—Ex (Cho et al. 2006; Mao et al. 2006; Silva et al. 2006; Willecke et al. 2008; Verghese et al. 2012b). D can reverse the effects of loss of *ft* on growth, and expression of Fat target genes like *wg*, *serrate*, and *fj* (Mao et al. 2006). Scrib was also placed upstream of D and Ex, and downstream of Ft based on genetic epistasis experiments (Verghese et al. 2012b). When Ft is inactive, D is regulated by Approximated (App) (Matakatsu and Blair 2008). App post-transcriptionally modifies D and affects its localization at the apical cell cortex. Hence, App functions in the Hippo pathway by affecting the availability of D at the apical cell cortex. When Ft is activated, D is released from App and binds to Zyxin (Zyx), which in turn interacts with Wts and stabilizes Wts activity (Rauskolb et al. 2011). Genetic epistasis experiments placed Zyx downstream of Ft and Dco, and upstream of Wts (Feng and Irvine 2007, 2009; Rauskolb et al. 2011). Thus, influencing Wts stability is a primary mechanism by which Ft controls growth via Hippo signaling. The other input via Ex remains less clear although there is clearly an input from Ft to Ex that also contributes to the Fat-branch-related phenotypes and regulation of the Hippo signaling pathway. Does Fat signaling simultaneously signal through Ex (and the core kinase cascade) and D; or the signals downstream of Ft are partitioned to allow maximum and more efficient signal transduction to the core kinase cascade remains unknown. Currently, the possibility that certain extracellular signals preferentially transmit the signal to Ex or D downstream of Ft has not been addressed.

II. Apical membrane proteins of the Hippo pathway Over the last 5 years, it has become clear that membrane-localized proteins are an intrinsic part of the Hippo signaling pathway (Genevet and Tapon 2011; Grusche et al. 2011; Halder et al. 2012; Schroeder and Halder 2012). Amongst these are the cell polarity proteins and proteins required for maintaining the cytoskeleton. The FERM (N-Terminal Globular domain (Band4.1, Ezrin, Radixin, Moesin)) domain-containing adaptor proteins Ex and Merlin (Mer) were amongst the earliest Hippo pathway components that were known to localize to the apical membrane (Hamaratoglu et al. 2006; McCartney et al. 2000). Ex and Mer act upstream of the Hpo kinase and regulate pathway activation (Hamaratoglu et al. 2006). Loss of *mer* and *ex* together in somatic clones caused dramatic overproliferation of cells leading to overgrowths. These effects were synergistic because loss of function of *ex* or *mer* alone does not cause similar defects. These genes function together to control proliferation by regulating expression of transcriptional targets of Hippo pathway (e.g., Cyclin E and DIAP1). Expanded can also regulate the pathway by independently interacting with Yki and sequestering it in the cytoplasm (Badouel et al. 2009; Oh et al. 2009).

Another protein that binds Ex and Mer, and acts upstream of Hpo is the WW- and C2-domain-containing adapter protein Kibra. Ex, Mer, and Kibra form a complex at the apical membrane in epithelial cells, which then activates the downstream core kinase cascade (Baumgartner et al. 2010; Cho et al. 2006; Genevet et al. 2010; Hamaratoglu et al. 2006; Pellock et al. 2007; Tyler and Baker 2007; Yu et al. 2010). Kibra was identified via a genome wide screen in *Drosophila* and in S2 cells for candidates that modified Yki activity (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010). Genetic epistasis experiments placed Kibra upstream of Hpo and Yorkie. Kibra affects the phosphorylation of Hpo and Yorkie. Kibra acts synergistically with Ex and Mer to regulate Wts phosphorylation, and Kibra binds to Sav, and Hpo in a Sav-dependent manner (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010).

Cell-polarity genes have been well characterized in flies and mammalian model systems, and recent studies reveals a role for cell polarity genes in the regulation of Hippo signaling (Genevet and Tapon 2011; Grusche et al. 2010; Grzeschik et al. 2007; Grzeschik et al. 2010a; Grzeschik et al. 2010b; Schroeder and Halder 2012). Crumbs (Crb), a trans-membrane protein is the upstream regulator that regulates Ex activity (Chen et al. 2010; Ling et al. 2010; Robinson et al. 2010). Crb is required for proper localization of Ex. Crb regulates Yki activity by interacting with Expanded (Chen et al. 2010; Grzeschik et al. 2010a; Robinson et al. 2010). Crb was found through a genetic screen, and loss and gain of function of Crb cause overgrowth of tissues and up-regulation of the Hippo pathway target genes. Echinoid (Ed) is another upstream regulator of the Hippo pathway, that like *kibra* interacts with both Ex and Yki (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010; Yue et al. 2012). Cells mutant for *ed* cause mislocalization of Sav from the subapical membrane without affecting Ex or Mer localization. Ed also interacts physically with Hpo, Ex, Mer, and Kibra (Yue et al. 2012).

F-actin acts as an upstream regulator of the Hippo pathway. Increased levels of F-actin inhibit the pathway and activation of Hippo pathway inhibits F-actin accumulation (Fernandez et al. 2011; Richardson 2011; Sansores-Garcia et al. 2011). Tao-1 phosphorylates Hpo at T195 and acts upstream of Hpo (Boggiano and Fehon 2012; Boggiano et al. 2011; Poon et al. 2011). RNAi knockdown of Kibra, Ex, and Mer resulted in a significant decrease of endogenous Hpo protein in the membrane fraction (Boggiano and Fehon 2012; Boggiano et al. 2011; Poon et al. 2011). Thus, the apical proteins regulate Hpo at least in part by bringing the latter to the membrane, where Hpo may be activated via mechanisms yet to be determined.

Negative Regulators of the Hippo Pathway

Several members of the Hippo pathway were identified based on their effects on tissue growth, and the loss of function phenotypes of these components showed dramatic outgrowths and benign lesions in fly epithelia. It was clear that additional components that keep this pathway in check (for example, phosphatases or kinase inhibitors) must exist, as Hippo activity would need to be modulated both positively and negatively for maintaining tissue homeostasis. Thus, the search for negative regulators began, which yielded many important and critical regulators of the Hippo pathway. Amongst the first genes identified in this category, was the Ras Association Family (RASSF) gene, *dRASSF1* (Polesello et al. 2006). The dRASSF protein negatively regulates the pathway by inhibiting the phosphorylation of Hpo, thus interrupting the Hpo kinase from signaling to the downstream kinase Wts (Polesello et al. 2006; Scheel and Hofmann 2003). Other inhibitors that act by dephosphorylating Hpo are the phosphatases—Striatin-interacting phosphatase and protein phosphatase 2A (PP2A) (Ribeiro et al. 2010). A second mechanism of inhibition of Yki activity was identified by the *Drosophila* Ajuba family gene, *djub* (Das Thakur et al. 2010). Loss of *djub* in mutant clones in imaginal discs caused reduced proliferation and increased apoptosis, akin to *yki* mutant clones. Genetic interaction studies showed that *djub* acts downstream of Hpo but upstream of Yki and Wts (Das Thakur et al. 2010). Furthermore, Djub can physically associate with Wts and Sav and influence the signaling activity of Yki. Thus, *djub* negatively regulates Hippo signaling by interfering with Yki phosphorylation and its subcellular localization (Das Thakur et al. 2010). Recently, another negative regulator, *myopic* (*mop*) was identified in a genetic screen for conditional growth suppressors (Gilbert et al. 2011). *mop* encodes the *Drosophila* homolog of human *His-domain protein tyrosine phosphatase* gene (*HD-PTP* or *PTPN23*) (Toyooka et al. 2000). *mop* mutant cells show overgrowth phenotypes due to a block in cell death. This growth is accompanied by upregulation of a subset of Yki transcriptional targets but not the antiapoptotic gene *diap1*. *mop* interacts genetically with *yki* and acts downstream of *wts*, but at the level of *ex* and *yki*. Myopic PPxY motifs bind conserved residues in the WW domains of the transcriptional coactivator Yorkie, and Myopic colocalizes with Yorkie at endosomes (Gilbert et al. 2011). Thus, several negative regulators of the Hippo pathway are now known; however, much remains unknown about their mechanism of action and their influence on growth regulation during development (Table 1).

Table 1 Regulators of the Hippo pathway

	Gene name, *symbol,* [Chr]	Nature of protein	Role	References
Upstream regulators	Crumbs *Crb* [3]	Protein kinase C binding	Organization of adherens junction, establishment of cell polarity, photoreceptor & rhabdomere development	Fan et al. 2003; Pichaud and Desplan 2002; Fan et al. 2003; Tepass et al. 1990
	Expanded *ex* [2]	Protein binding	Compound eye, photoreceptor cell differentiation, negative regulation of hippo signaling cascade	Maitra et al. 2006; Pellock et al. 2007; Badouel et al. 2009; McCartney et al. 2000
	Merlin *Mer* [1]	Protein binding	Regulation of programmed cell death, negative regulator of hippo signaling	Pellock et al. 2007; Hamaratoglu et al. 2006
	Kibra *Kibra* [3]	Protein binding	Compound eye morphogenesis, regulation of hippo signaling cascade	Ling et al. 2010; Genevet et al. 2010; Yu et al. 2010; Baumgartner et al. 2010
Fat branch	Fat *ft* [2]	Cell adhesion molecule binding	Establishment of planar polarity, negative regulation of growth, imaginal disc growth	Yang et al. 2002; Mao et al. 2006; Torok et al. 1993; Garoia et al. 2000; Matakatsu and Blair 2006
	Lowfat *lft* [2]	Protein binding	Wing morphogenesis	Mao et al. 2009
	Dachs *D* [2]	ATPase activity (predicted nature)	Establishment of ommatidial planar polarity, positive regulation of growth	Mao et al. 2006
	Dachsous *Ds* [2]	Cell adhesion molecule binding	Eye morphogenesis, establishment of cell polarity, cell proliferation	Baena-Lopez et al. 2005; Clark et al. 1995
	Four-jointed *Fj* [2]	Wnt-protein binding; protein kinase activity	Imaginal disc growth, establishment of planar polarity	Villano and Katz 1995; Bosveld et al. 2012
	Scribbled *Scrib* [2]	Protein binding	Establishment of ommatidial planar polarity, negative regulation of imaginal disc growth	Courbard et al. 2009; Zeitler et al. 2004; Verghese et al. 2012a
	Zyxin *Zyx* [4]	Protein binding	Positive regulation of imaginal disc growth	Rauskolb et al. 2011
	Approximated *App* [3]	Protein-cysteine S-palmitoleyltransferase activity (predicted nature)	Establishment of body hair or bristle planar orientation	Matakatsu and Blair 2008
	Discs Overgrown *Dco* [3]	Kinase activity	Establishment of ommatidial planar polarity, positive regulation of cell growth	Strutt et al. 2006; Klein et al. 2006; Guan et al. 2007

Table 1 (continued)

	Gene name, *symbol,* [Chr]	Nature of protein	Role	References
Core kinase cascade	Warts *Wts* [3]	Protein binding, kinase activity	Negative regulation of cell proliferation, R8 cell fate specification	Justice et al. 1995; Mikeladze-Dvali et al. 2005
	Mob as tumor suppressor *Mats* [3]	Protein binding	Cell proliferation	Lai et al. 2005
	Hippo *Hpo* [2]	Protein binding; serine/threonine kinase activity	Negative regulation of cell proliferation, R8 cell fate specification	Udan et al. 2003; Mikeladze-Dvali et al. 2005
	Salvador *Sav* [3]	Protein binding	Negative regulation of cell proliferation, R8 cell fate specification	Kango-Singh et al. 2002; Mikeladze-Dvali et al. 2005
Other regulators	Ajuba *Jub* [1]	Ligand-dependent nuclear receptor binding	Positive regulation of organ growth	Das Thakur et al. 2010
	Tao *Tao* [1]	Serine/threonine kinase activity	Negative regulation of organ growth	Poon et al. 2011
	Echinoid *Ed* [2]	Protein binding	Negative regulation of hippo signaling cascade	Yue et al. 2012
	Pez *Pez* [2]	Protein tyrosine phosphatase activity	Negative regulation of hippo signaling cascade	Poernbacher et al. 2012
	d-STRIPAK PP2A *Pp2A-29B* [2]	Serine/threonine phosphatase activity	Centrosome organization	Dobbelaere et al. 2008
	Ras association family member *Rassf* [3]	Protein binding	Negative regulation of signal transduction	Polesello et al. 2006
	Par-6 *Par-6* [1]	Protein binding	Cell adhesion	Kiger et al. 2003
	Atypical protein kinase C *a-PKC* [2]	Protein binding; serine/threonine kinase activity	Compound eye retinal cell programmed cell death, establishment of epithelial cell planar polarity	Ogawa et al. 2009; Kaplan et al. 2011
	Stardust *Sdt* [1]	Protein binding	Zonula adherens assembly	Nam and Choi 2003; Bachmann et al. 2001

Table 1 (continued)

	Gene name, *symbol,* [Chr]	Nature of protein	Role	References
	Lethal 2 giant Larvae *L2gl* [2]	Myosin II binding; myosin binding	Cell competition in a multicellular organism, establishment of epithelial cell planar polarity	Tamori et al. 2010; Kaplan and Tolwinski 2010
	Myopic *Mop* [3]	Protein tyrosine phosphatase activity	Regulation of growth	Gilbert et al. 2011
	Patj *dPatj* [3]	Protein kinase C binding	Adherens junction organization	Nam and Choi 2006
Transcription factors / co-activators	Yorkie *Yki* [2]	Protein binding; transcription co-activator activity	Cell competition in a multicellular organism, cell proliferation	Ziosi et al. 2010; Huang et al. 2005; Thompson and Cohen 2006
	Scalloped *Sd* [1]	Transcription factor binding	Compound eye morphogenesis	Garg et al. 2007
	Homothorax *Hth* [3]	Protein binding; transcription factor	Compound eye photoreceptor fate determination	Wernet et al. 2003
	Teashirt *Tsh* [2]	Transcription factor activity	Eye-antennal disc development	Singh et al. 2004
	Wpb2 *Wbp2* [3]	Transcription factor binding	Positive regulation of imaginal disc growth	Zhang et al. 2011b
	Mothers against dpp *Mad* [2]	Transcription factor activity	Compound eye morphogenesis, negative regulation of gene expression	Cordero et al. 2007; Anderson et al. 2006

Hippo Pathway Cross-talks With Other Pathways

Hippo pathway is known to interact with other pathways to regulate growth. In mice it has been shown that Mst2 interacts with Raf-1 of the ERK/MAPK pathway (Graves et al. 1998). Raf-1 inhibits dimerization of Mst2 and recruits a phosphatase to dephosphorylate Mst2, thereby inactivating it, a function independent of the MAPK pathway (O'Neill and Kolch 2005). More recently, many points of intersection between Hippo and other signaling pathways have come to light. For example, in the last 5 years, Hippo pathway was shown to interact with JNK pathway to regulate compensatory proliferation, regeneration, and tumor progression (Chen et al. 2012; Doggett et al. 2011; Grzeschik et al. 2010a; Staley and Irvine 2010; Sun and Irvine 2010, 2011; Tyler et al. 2007; Varelas et al. 2010a). Furthermore, Hippo pathway interacts with Wingless/Wnt pathways in flies and mammals (Varelas et al. 2010a). Hippo pathway restricts Wnt/beta-Catenin signaling by promoting an interaction between TAZ and dishevelled (DVL) in the cytoplasm. TAZ inhibits the CK1delta/epsilon-mediated phosphorylation of DVL, thereby inhibiting Wnt/beta-Catenin signaling (Azzolin et al. 2012; Tsai et al. 2012; Varelas et al. 2010a). In *Drosophila*, Hippo signaling modulates Wg target gene expression (Varelas et al. 2010a). More connections of Hippo signaling with pathways that control morphogenetic patterning and growth have been uncovered which include the discovery of the regulation of TGF-beta Transforming Growth Factor/SMAD (refers to a family of transcription factors: Sma from *Caenorhabditis elegans*, Mad 1 from *Drosophila,* and SMAD1 from human) complexes by YAP/TAZ (transcriptional co-activator with PDZ) in mammalian models and Yki in flies (Chan et al. 2011; Meignin et al. 2007; Polesello and Tapon 2007; Rogulja et al. 2008; Sudol and Harvey 2010; Varelas et al. 2010b). Dpp (Decapentaplegic) signaling interacts with D to maintain Fj and Ds gradient in order to regulate proliferation in the wing (Rogulja et al. 2008). Hippo pathway also intersects the phosphoinositide 3-kinase (PI3K)/TOR pathway via multiple interactions (Bellosta and Gallant 2010; Collak et al. 2012; Karni et al. 2008; Kim et al. 2010; Mills et al. 2008; Sekido 2008; Strassburger et al. 2012; Tumaneng et al. 2012a; Tumaneng et al. 2012b; Wehr et al. 2012); with G-protein coupled receptor signaling (Yu et al. 2012) and Receptor Tyrosine Kinase signaling (Gadd et al. 2012; Garami et al. 2003). In fact, the web of interactions has grown exponentially over the last few years such that oftentimes the Hippo pathway is referred to as a network or superhighway (Barry and Camargo 2013; Table 2).

Mammalian Hippo Pathway

In vertebrate models, Hippo pathway is responsible for regulating organ size, and is involved in regeneration (Bertini et al. 2009; Hiemer and Varelas 2013; Hong and Guan 2012; Liu et al. 2012a). The core kinase pathway is highly conserved in mammals (Hong and Guan 2012; Liu et al. 2012a; Zhao et al. 2008a), and consists of Mst1/2 (Hpo homolog) and LATS1/2 (Wts homolog) along with their adaptor proteins WW45 (Sav) and MOB1 (Mats homolog), which control growth by regulating

Table 2 Pathways known to interact with the Hippo network

Pathway interactions	Responses	References
JNK pathway	Cell-competition, compensatory proliferation, regeneration, cytoskeletal integrity, tumorigenesis	Chen et al. 2012; Sun et al. 2011; Densham et al. 2009; Enomoto et al. 2011
Wingless pathway	Growth control	Varelas et al. 2010a
EGFR pathway	Growth control	Herranz et al. 2012
Decapentaplegic pathway	Growth control	Rogulja et al. 2008
Hedhehog pathway	Growth control, neuronal differentiation	Kagey et al. 2012; Lin et al. 2012
Notch pathway	Neural stem-cell maintenance, polar cell fate during oogenesis, cell-differentiation, proliferation	Li et al. 2012; Chen et al. 2011; Yu et al. 2008
TSC-TOR pathway		Wehr et al. 2013; Tumaneng et al., 2012a, b; Strassburger et al., 2012

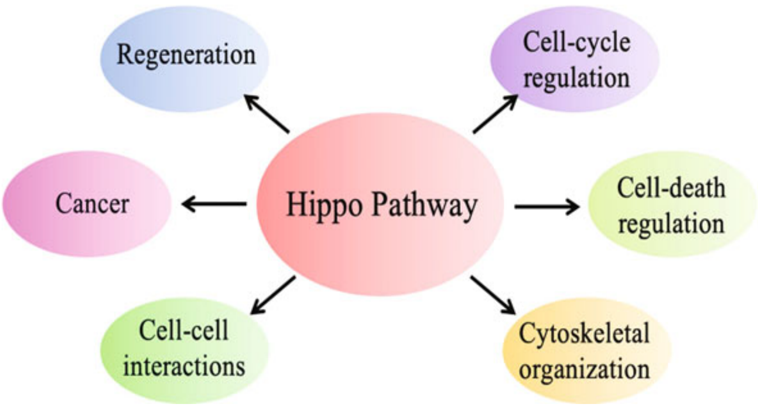

Fig. 4 Hippo pathway is linked to many biological and developmental processes. Hippo signaling has been shown to participate in generating myriad cellular responses that are aimed at attaining tissue homeostasis in addition to regulating organ size. Thus, the role of Hippo signaling is implicated not only during organ development but also in differentiated tissues. Further, tumorigenesis has also been attributed to dysregulation of Hippo signaling pathway placing it in the global network of regulatory mechanisms required for proper growth

phosphorylation of YAP (Yki homolog) and TAZ (transcriptional coactivator with PDZ-binding domain); Hong and Guan 2012; Liu et al. 2012a; Zhao et al. 2008a). Ft1-4 (Ft homolog), Dchs1-2 (Ds homolog), and Fjx1 (Fj homolog) are known to regulate PCP; however, their connection to other Hippo pathway components still needs to be explored (Brittle et al. 2010; Hiemer and Varelas 2013; Skouloudaki et al. 2009; Sopko et al. 2009; Zhao et al. 2007). The other downstream components like Dco and Lowfat homolog have not been shown yet to function within the Hippo pathway (Sopko et al. 2009; Zhang et al. 2008a, 2011a; Zhao et al. 2010a). However, Dco homolog CK1δ/ε has been shown to be involved in YAP/TAZ degradation (Zhao et al. 2010b).

Neurofibromatosis type II (NF2), the Mer homolog is the most extensively studied upstream regulator in mammals (Sekido 2011; Striedinger et al. 2008; Zhang et al. 2009b; Zhao et al. 2007). NF2 interacts with CD44 and adherens junction to relay the signal downstream to other Hippo pathway components during contact inhibition (Li et al. 2012; Morrison et al. 2001; Zhao et al. 2007). Kibra is known to interact with LATS2 to promote its phosphorylation (Zhang et al. 2012). It also protects LATS2 from proteosomal degradation by preventing its ubiquitination. Kibra is also the transcriptional target of Hippo pathway (Angus et al. 2012; Ishiuchi and Takeichi 2012; Visser-Grieve et al. 2012; Xiao et al. 2011). Angiomotin family interacts with its PPxY domain to YAP WW domain and TAZ PDZ domain independent of the upstream components. This interaction inhibits the activity of YAP/TAZ (Chan et al. 2011; Paramasivam et al. 2011; Skouloudaki and Walz 2012; Wang et al. 2012a; Wang et al. 2009; Zhao et al. 2011a). Ex1/FRMD6/Willin (Ex homolog) interacts with upstream Hippo pathway components like Mer (Angus et al. 2012; Ishiuchi and Takeichi 2012; Visser-Grieve et al. 2012). Crb interacts with YAP/TAZ and promotes its phosphorylation, which is dependent on cell density and at the same time inhibits TGF-beta SMAD pathway (Varelas et al. 2010b). Unlike *Drosophila* RASSF1, mammalian RASSF homologs activate MST1/2 (Avruch et al. 2012; Guo et al. 2007; Hergovich 2012; Hwang et al. 2007; Polesello et al. 2006; Ribeiro et al. 2010; Schagdarsurengin et al. 2010; Seidel et al. 2007).

Nephronophthisis4 (NPHP4), a known cilia-associated protein that is mutated in the severe degenerative renal disease nephronophthisis, acts as a potent negative regulator of mammalian Hippo signaling (Habbig et al. 2011, 2012). NPHP4 directly interacts with the kinase LATS1 and inhibits LATS1-mediated phosphorylation of the YAP and TAZ, leading to derepression of these proto-oncogenic transcriptional regulators. Moreover, NPHP4 induces release of YAP and TAZ from 14-3-3 binding and their nuclear translocation promoting TEA domain (TEAD)/TAZ/YAP-dependent transcriptional activity (Habbig et al. 2011). ITCH interacts with LATS to negatively regulate its stability (Ho et al. 2011; Salah et al. 2011; Wang et al. 2012a). α-catenin interacts with YAP and affects its stability by stabilizing the YAP/14-3-3 complex to restrict YAP activity, and by preventing PP2A to interact with YAP (Azzolin et al. 2012; Schlegelmilch et al. 2011; Silvis et al. 2011; Tsai et al. 2012; Varelas 2010a; Konsavage 2013; Mauviel 2012). Zona occludens-2 (ZO-2) promotes the pro-apoptotic function of YAP (Oka et al. 2010). The ASPP (apoptosis-stimulating protein of p53) family of proteins can function in the nucleus to modulate the transcriptional activity of p53, with ASPP1 and ASPP2 contributing to the expression of apoptotic target genes (Vigneron et al. 2010). ASPP increases YAP/TAZ nuclear availability by preventing LATS interaction with YAP/TAZ (Vigneron et al. 2010). Similarly, PP1A interacts with ASPP1 to dephosphorylate TAZ leading to increased TAZ nuclear availability (Liu et al. 2010, 2011).

In mammalian cell lines, E-cadherin acts as an upstream regulator of the pathway, which activates the pathway in response to contact inhibition. YAP and TAZ interact with several transcriptional factors. YAP/TAZ interacts with TEAD1/4 and Runx2. TAZ interacts with thyroid transcription factor-1, peroxisome proliferator-activated receptor gamma (PPARγ), Tbx5, Pax3, and Smad2/3/4. Yap interacts with p73 to mediate its pro-apoptotic functions. Various target genes are as follows: *CTGF*, *AREG*, *BIRC5–2*, *GLI-2* (Liu et al. 2012b; Zhao et al. 2008a, 2010a). YAP1 interacts with Sonic Hedgehog pathway to promote the proliferation of cerebellar granule neuron precursors (CGNPs). TAZ inhibits Wnt signaling by inhibiting the phosphorylation of DVL by CKIδε. YAP/TAZ has also been shown to interact with SMAD to regulate tumorigenesis (Zhang et al. 2011a, b). Thus, our understanding of the mammalian Hippo pathway continues to grow with new insights on its molecular and signaling interactions with components from Hippo and other pathways.

The Insulin-Receptor Signaling Pathway: Regulation of Cell Size

The pin-head screens showed a large number of mutations that primarily caused decreased growth due to formation of smaller cells (Oldham et al. 2000a; Stocker and Hafen 2000). These mutants were subsequently categorized into two well-studied signaling pathways—the insulin/Phospho inositol 3 kinase (PI3K) pathway and the Target of Rapamycin (TOR) pathway. Using genetic and biochemical strategies, the epistatic and molecular interactions were elucidated for genes that comprise these pathways.

The Regulation of Cell Size and Not Cell Numbers

The PI3K Pathway *Drosophila* has one insulin/insulin-like growth factor (IGF) receptor homolog known as dINR (*Drosophila* insulin receptor) (Chen et al. 1996; Fernandez et al. 1995), and several insulin-like peptides (Brogiolo et al. 2001). These together control the carbohydrate metabolism and growth in flies (Ikeya et al. 2002; Rulifson et al. 2002). Through a mechanism that involves phosphorylation of its carboxy-terminal end, the dINR recruits downstream signaling molecules without the need for adaptor proteins. The signaling also involves the insulin receptor substrate protein Chico, which contains a phosphotyrosine binding domain, which facilitates its binding with activated dINR (Bohni et al. 1999; Poltilove et al. 2000). Subsequently, the pathway functions by activating the PI3K pathway, via activation of the Drosophila PI3K—Dp110 and its adapter subunit Dp60 (Leevers 2001; Leevers et al. 1996; Weinkove et al. 1999). Dp110/Dp60 heterodimers are recruited to the plasma membrane following the binding of p60 SH2 domain to phosphorylated dINR and Chico, which allows the PI3K access to the phosphinositide substrates in the plasma membrane. This sets up a signaling cascade in which PIP3 (phosphatidyl inositol (3, 4, 5 triphosphate)) transduces the signal to downstream effectors that contain the PIP3-binding PH (Pleckstrin homology) domains, and causes relocalization of these proteins to the plasma membrane.

In flies, two such effectors exist, which are the *Drosophila* homolog of phosphoinositide-dependent kinase 1 (PDK1) and its substrate AKT (AK: mouse strain that develops thymic lymphomas; T: thymoma) aka protein kinase B (PKB). PDK1 localizes to the membrane during low levels of PI3K activity via its affinity to PIP3, whereas AKT requires high levels of PI3K activity to become membrane localized, through a process involving binding of PIP3 to its PH-domain and phosphorylation by PDK1 (Vanhaesebroeck and Alessi 2000). In flies, the activity of dAKT is reduced in the absence of Dp110 and coexpression of dPDK1 and dAKT activates dAKT and induce growth (Cho et al. 2001; Radimerski et al. 2002b; Rintelen et al. 2001).

A negative regulator of the PI3K activity is the lipid phosphatase PTEN (phosphate and tensin homolog), which removes the 3' phosphate from three phosphoinositides generated by PI3K (Gao et al. 2000; Goberdhan et al. 1999; Huang et al. 1999). Genetic interaction studies support the model where PTEN directly antagonizes PI3K. Loss of PTEN leads to overgrowths due to increased levels of PIP3 (Oldham et al. 2002). Recently, the FOXO (Forkhead box)family of transcription factors was identified as the target that enabled AKT to regulate growth (Tran et al. 2003). AKT-mediated phosphorylation of FOXO antagonizes its transcriptional activity by creating a 14-3-3 binding site that leads to cytoplasmic sequestration of FOXO (Brunet et al. 1999, 2002; Burgering and Kops 2002). *Drosophila* has one FOXO family transcription factor (dFOXO)—which functions downstream of AKT. Interestingly, loss of function of dFOXO has no apparent effect on cell size or growth as flies homozygous mutant for dFOXO are viable and normal in size (Junger et al. 2003).

The loss of function of Dp110, p60, chico, dINR, dPDK1, and dAKT show similar effects on cell size and tissue growth. For example, twin-spot analysis revealed that loss of function clones of mutations in these genes are smaller than the corresponding wild-type twin clones that lead to formation of smaller structures (Bohni et al. 1999; Brogiolo et al. 2001; Rintelen et al. 2001; Verdu et al. 1999; Weinkove et al. 1999). Overexpression of PI3K pathway components like Dp110 leads to increased insulin/PI3K signaling and a corresponding increase in cell size, cell number, and tissue growth (Goberdhan et al. 1999; Huang et al. 1999; Leevers et al. 1996). Overall, changes in levels of insulin/PI3K signaling have profound effects on organ and organismal size due to effects on cell growth and cell division throughout development and affect the final body/organ size (Fig. 5).

The TSC-TOR Pathway

Two TOR genes, *TOR1* and *TOR2*, were initially identified in yeast, and were shown to be kinases that regulate growth in all organisms by acting as nutrient sensors that couple signaling to nutrient availability (Gingras et al. 2001). *Drosophila* TOR (dTOR) promotes growth by stimulating translation via promoting the activity of the *Drosophila* S6Kinase (Montagne et al. 1999), and inhibiting the *Drosophila* 4E-BP1 (a homolog of the Eukaryotic translation initiator 4E)—the translational inhibitor of eIF4E, which is a part of the translation initiation complex (Gingras et al. 2001; Lasko 2000). Hyperphosphorylation of d4E-BP1, which is in part controlled by the TOR kinase, relieves its interaction with eIF4E leading to translation initiation.

TOR signaling is negatively regulated by a complex formed by the tuberous sclerosis complex tumor suppressors, TSC1 and TSC2 (Marygold and Leevers 2002).

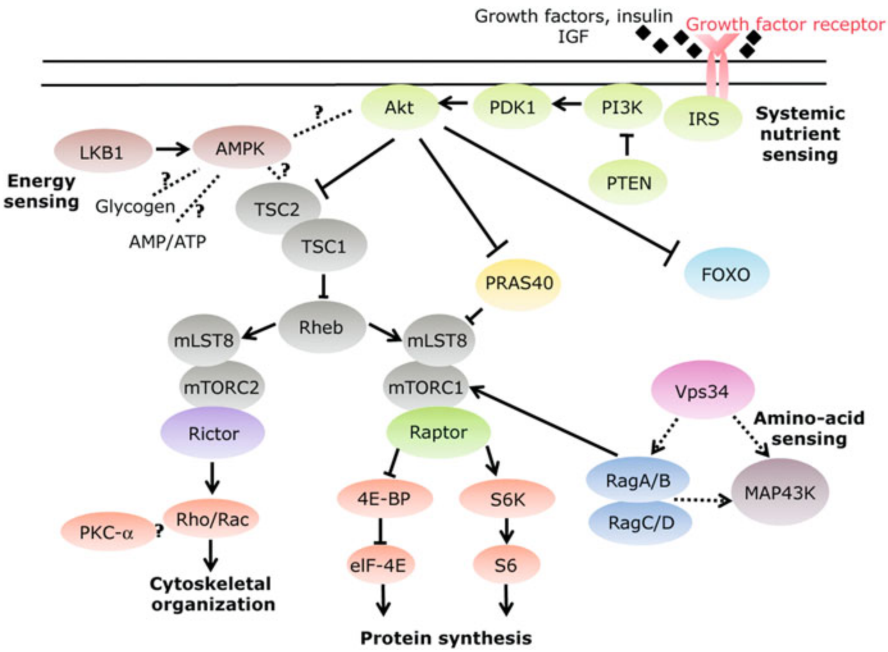

Fig. 5 Model depicting regulation of INR/TOR signaling pathway governed by nutritional status in *Drosophila*. Cellular growth in part is also dependent on the availability of nutrients. This aspect of growth regulation is mainly regulated by the insulin/TOR signaling pathway. Some of the well-studied players of the pathway include phosphatidylinositide 3-kinase and Akt that integrate upstream signaling from growth factor receptors and relay it to TSC1 and TSC2 to regulate ribosomal and protein biosynthesis in addition to actin organization. Other energy sensing and amino-acid sensing mechanisms are also thought to interact with the core TSC/TOR pathway. However, the exact role or the mechanism by which this takes place remains largely unknown

Mutations in *Tsc1/Tsc2* cause formation of large cells, and are implicated in the inherited benign hamartomas observed in the tuberous sclerosis patients (Kandt 2002; Montagne et al. 2001). The *Drosophila Tsc1/2* genes show similar effects on cell size, and were identified by several groups in the *eyFLP cell lethal* screens as mutants with overgrown heads (Gao and Pan 2001; Potter et al. 2001; Tapon et al. 2001). Loss of TSC1/2 causes increased growth whereas overexpression of TSC1/2 causes reduced growth due to slow cell cycle progression in the mutant cells. Growth regulation via TSC1/2 happens through preventing dS6K activation via dTOR (Gao et al. 2002; Radimerski et al. 2002a; Radimerski et al. 2002b). Another important component of this pathway is the GTPase (guanosine triphosphate hydrolase) *Rheb*, which is a target of TSC (Saucedo et al. 2003; Stocker et al. 2003; Zhang et al. 2003). The Rheb-GTP levels play a central role in regulating the activity of TOR pathway, and the TOR protein that exists in two large multimeric complexes in the cell, viz., the rapamycin-sensitive TORC1 complex, and the rapamycin-resistant TORC2 complex (Hara et al. 2002; Kim et al. 2002; Kim et al. 2003; Loewith et al. 2002; Sarbassov et al. 2004).

The TORC1 complex consists of TOR, Raptor, and GßL, and responds to the presence of growth factors and nutrients to control protein synthesis. The small GTPase protein Rheb (Ras homolog enriched in brain) is a direct activator of TORC1 (Long et al. 2004; Saucedo et al. 2003; Stocker et al. 2003), and the TSC complex (TSC1/TSC2) negatively regulates TORC1 by functioning as a GTPase-activating protein for Rheb (Potter and Xu 2001; Zhang et al. 2003). Growth factors such as insulin or IGFs activate TORC1 signaling upstream of the TSC1/TSC2 (TSC1/2) complex through the insulin receptor (InR)/PI3K/AKT signaling pathway (Inoki et al. 2002; Potter et al. 2002). TORC1 also senses nutrient availability. Amino acids regulate TORC1 through mechanisms independent or downstream of TSC complex, and recently the Rag small GTPases have been shown to interact with TOR and promote TORC1 activity by controlling its subcellular localization (Nellist et al. 2008; Sancak et al. 2010).

TORC2 complex consists of TOR, rictor, Sin1 (stress-activated map kinase-interacting protein 1) and GßL; and phosphorylates and activates several AGC family kinases, including AKT, serum and glucocorticoid-regulated kinase (SGK), and protein kinase C, and thereby regulates cell survival, cell cycle progression, and metabolism (Pearce et al. 2010; Li 2010; Gao 2010). In contrast to TORC1, little is known about the upstream activators of mTORC2. Although the general mechanisms have not been accepted, PI3K, TSC, and Rheb have been shown to regulate TORC2 activity, and Rictor has been identified as a substrate of S6 kinase (S6K), suggesting possible regulation of TORC2 through the TORC1 pathway (Dibble et al. 2009; Treins et al. 2010; Yang et al. 2006). Nevertheless, it is generally thought that growth factors may control TORC2, either directly or indirectly (Zinzalla et al. 2011). TORC2 has been proposed to function independent of amino acid availability (Jacinto et al. 2006); however, recent findings show that amino acids may also activate TORC2 (Tato et al. 2011).

The central role of TOR in cell growth has been largely attributed to TORC1, but mounting evidence points to a role for TORC2 as well in this basic cellular process. For instance, TORC2 localizes in polysomal fractions and associates with ribosomal proteins, indicating a potential role for TORC2 in protein synthesis and maturation (Cybulski and Hall 2009; Zinzalla et al. 2011). *lst8* knockout flies are viable but small, similar to *rictor* mutants but dissimilar to files with *tor* or *rheb* mutations, which are lethal (Avruch et al. 2009; Liao et al. 2008; Wang et al. 2012b). Neither loss nor overexpression of LST8 affected the kinase activity of TORC1 toward S6K or autophagy, whereas the kinase activity of TORC2 toward AKT was completely lost in the *lst8* mutants (Avruch et al. 2009; Liao et al. 2008; Wang et al. 2012b).

In terms of effects of TOR signaling on growth phenotypes in *Drosophila*, loss of dTOR leads to a decrease in larvae size; however, the larvae fail to mature and die before reaching adulthood. In mosaic *Drosophila*, loss of dTOR leads to a decrease in cell size while maintaining the general organization of the tissue (Oldham et al. 2000b; Zhang et al. 2000). However, it is less clear how cell size is regulated downstream of mTOR. One of the most potent candidates in this regulation is S6K. In *Drosophila*, knockout of *S6K* results in high rates of embryonic lethality. In the surviving adults, however, there is a decrease in body size. Knockdown of either dPTEN or dTSC1is sufficient to increase cell size; however, a double knockdown of dPTEN and dTSC1 has additive effects on cell size regulation. This suggests that in *Drosophila*, the pathways may have independent components in the regulation of cell size (Gao and Pan 2001). It may also highlight the differences in the regulation of TSC2 by AKT in *Drosophila* as seen by mutations of the AKT phosphorylation sites on TSC2 (Dong and Pan 2004; Pan et al. 2004). Loss of either dPTEN or dTSC1 can lead to increase in cell size; however, a report has suggested that only knockdown of dTSC1 leads to increase in dS6K (Radimerski et al. 2002a), whereas other reports have also seen increases in dS6K with the knockdown of dPTEN (Sarbassov et al. 2004; Yang et al. 2006). It is possible that dTSC1 regulates cell size in a dTOR-dependent manner, whereas dPTEN partially regulates cell size in a dTOR-independent manner (Radimerski et al. 2002b).

In conclusion, the TOR signaling pathway is a complex network of cell size regulators that is also implicated in tumorigenesis and cell survival. Several pathways interact and intersect with the TOR pathway at multiple points upstream and downstream of TOR.

Growth Regulation: A Network of Tumor Suppressors

Overall, growth control occurs through the Hippo and TSC-TOR pathways in conjunction with pathways regulating pattern formation during development. These pathways intersect in complicated signaling networks in all cell types, and coordinately regulate overall growth of an organism. Our progress in understanding of these pathways has lead the way to find molecules and interactions important for regenerative growth and wound healing—phenomena that have been well-documented but not well-understood at the molecular level for a long time. In addition, the establishment of these growth regulatory networks has generated many insights in the fields of cancer (e.g., the underlying genetics and biology link between hamartomas and TSC genes; Schwannomma's and NF2; YAP and Hepatocellular carcinoma; TAZ and Breast cancer etc.). In the future, it will be interesting to learn about the regulation of these pathways by extracellular and intracellular mechanisms, an area expected to expand rapidly with our increased understanding of the integration points in the circuitry of these networks.

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