GROWTH CONTROL BY HIPPO AND AKT SIGNALING PATHWAYS
IN DROSOPHILA MELANOGASTER

A Dissertation in
Genetics
by
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Coordinated cell proliferation, cell growth and cell death are required to control normal organ size in multicellular organisms. The perturbations of the mechanisms that regulate normal tissue growth are commonly found in cancers. A novel signaling pathway, Hippo tumor suppressor pathway, was identified in *Drosophila*. Loss of Hippo signaling leads to tissue overgrowth by promoting cell proliferation, cell growth and inhibiting apoptosis. Yorkie (Yki), the downstream effector of Hippo pathway, functions as a transcription co-activator to mediate growth control. In this study, I present that transcription factor Scalloped (Sd) is a critical partner of Yki to mediate Yki-dependent biological functions. Yki and its mammalian homolog, YAP, both contain one or two WW domains, which function as interaction module by binding to PPxY motifs. Functional analyses revealed that both WW domains confer a positive role in Yki/YAP-dependent growth promoting activity. I also show that crosstalk exists between Hippo and Akt signaling pathways. Akt signaling promotes cellular growth and cell division primarily via activation of biosynthesis. Activation of Akt pathway in loss of Hippo signaling context might be utilized to promote cellular growth in coordination with accelerated cell proliferation.
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Chapter 1

Introduction

1.1 Organ Size Control in Metazoans

Development of organs in multicellular organisms includes the control of size and patterning. Both processes need the intrinsic regulatory mechanisms in response to extrinsic cues, such as nutrients, hormones and crowding, etc. “Patterning” topic has long drawn the interests of developmental biologists and a number of signaling transduction pathways that regulate cell fate decisions have been identified, including BMP/TGF-beta, Wnt, Hedgehog (Hh), Notch, and EGF (Neto-Silva et al., 2009). It was in recent years that the attention was directed to investigate the internal mechanisms that control organ size (Pan, 2007).

The final size of organs is affected by three major cellular behaviors: cell growth, cell division and apoptosis. Cell growth refers to the mass accumulation of individual cells. The best known signaling pathway that regulates cell growth is the Insulin/TOR (target of rapamycin) pathway (Grewal, 2009). Insulin/TOR signaling influences ribosome synthesis, mRNA translation, autophagy and endocytosis in response to nutrient availability, stress and aging.

Cell division and programmed cell death (apoptosis) together determine the cell number. A novel signaling pathway, Hippo pathway, coordinately couples these two processes. Loss of the tumor suppressor components of Hippo pathway leads to increased cell proliferation and reduced apoptosis, which in turn causes tissue overgrowth and eventually tumor formation in Drosophila and mammals (Pan, 2007).
1.2 Genetic Basis for Tumorigenesis

Cancer is a disease of gene mutations, with a group of genetically heterozygous disorders. There is no common genetic make-up for all tumors. Instead, each individual tumor has its unique genetic signature. However, the link between them is the disturbance of complex signaling networks, which normally mediate the intra- or inter-cellular communications in multicellular organisms. Mutations of the components of cell signaling networks, which are usually oncogenes or tumor suppressor genes, play an important role in tumorigenesis.

Oncogenes normally promote cell growth and proliferation. Gain-of-function oncogenic mutations lead to increased cell proliferation and cell survival and potentiate the formation of tumors (Croce, 2008). Think of a cell as a car, an oncogene normally functions as the gas pedal, which promotes the cell to grow and divide. When the oncogenic mutation occurs, comparable to the gas pedal getting stuck down, the cell divides and grows out of control.

On the other hand, tumor suppressor genes include but are not limited to negative regulators of cell cycle progression, components in apoptosis, DNA damage repair and mitogenic signaling (Sherr, 2004). Inactivating mutations of tumor suppressor genes accelerate cell growth and lead to cancer, similar to the effects of dysfunctional brake in the car. There are two kinds of mutations in tumor suppressor genes (Bryant et al., 1993). Neoplastic mutations of tumor suppressors disrupt the tissue morphology and compromise cell differentiation in addition to causing tissue overgrowth, such as the apicobasal polarity regulators, Scribbled (Scrib), Discs large (Dlg) and Lethal (2) giant larvae (Lgl) (Humbert et al., 2008). Hyperplastic mutations, however, refer to the mutations of tumor suppressors that cause tissue overgrowth without affecting normal tissue architecture and differentiation, such as the components of the novel Hippo signaling pathway, which I will discuss in details below (Reddy and Irvine, 2008).
1.3 Drosophila as Cancer Research Model

To address the fundamental questions of cancer biology, a simpler multicellular organism, such as Drosophila, works as a powerful and efficient model system (Vidal and Cagan, 2006). With abundant genetic information and powerful tools available, researchers are able to investigate the functions of oncogenes and tumor suppressors with single cell resolution in the context of entire live animal. Moreover, large scale genetic screen in Drosophila has identified numerous genes critical for normal development as well as tumorigenesis.

The genes playing important roles during development are usually essential genes and their homozygous animals are lethal, which makes it difficult to dissect their precise functions in a given biological process. The introduction of the yeast site-specific FLP/FRT recombination system into Drosophila established the leader role of this model in understanding cell cycle mechanisms and cell signaling pathways (Golic and Lindquist, 1989). Dominant cell marker together with a proximal FRT site is introduced into the chromosome, homologous to the other chromosome carrying a proximal FRT site and a distal gene mutation (Xu and Rubin, 1993). Upon heat shock-driven, or temporal/spatial-specific expression of FLP recombinase, somatic recombination occurs between those two homologous FRT sites, generating one daughter cell homozygous for the cell marker and another daughter cell homozygous for the gene mutation (Figure 1-1).

Each cell will divide and generate a clone of cells with the same genetic background. Instead of interpreting the gene function during development based on its terminal adult cuticular phenotype, which is not precise and might be even misleading in some cases, the cell-autonomous marker makes it possible to analyze the gene effects throughout the different developmental stages. Generation of clones of cells with target gene mutation in the context of wild type neighbors in this way can be utilized to mimic several aspects of tumor biology, such as over-proliferation, apoptosis inhibition, cell competition and metastasis. Since most solid tumors occur within the epithelial cells, imaginal discs of Drosophila larvae, the intact dividing epithelia, provide an excellent model to generate homozygous mutant clones.
Figure 1-1: FLP/FRT genetic mosaic analysis. Parental cells are heterozygous for target gene mutation and cell marker (light green stands for one copy of marker gene). Upon FLP expression, mitotic recombination occurs between two FRT sites (white arrow head) on nonsister chromatids. Two daughter cells homozygous for either target gene mutation or cell marker (dark green stands for two copies of marker gene) are generated. Each individual cell divides and grows into a clone of cells.

In addition to its application in basic research, *Drosophila* is also used to generate specific disease models, including some cancer syndromes such as neurofibromatosis type 1 (NF1) and multiple endocrine neoplasia types I and 2 (MEN1 and MEN2, respectively) (Busygina et al., 2004; Hackstein, 1992; Read et al., 2005). Another growing trend is to use *Drosophila* as *in vivo* model to screen drugs. It provides a rapid and cheap animal model to identify lead compounds for treatment of human diseases (Workshop on 51th *Drosophila* Conference).

1.4 Discovery History of Hippo Signaling Pathway

The mechanisms underlying developmental growth control have remained poorly understood until the discovery of a novel tumor suppressor signaling pathway, Hippo
pathway (Reddy and Irvine, 2008). The genes, whose inactivating mutations result in excessive tissue growth, are termed tumor-suppressors (Hariharan and Bilder, 2006). A novel tumor suppressor signaling pathway, Hippo signaling pathway, has been recently identified in *Drosophila* (Figure 1-2). The Hippo pathway regulates cell growth, cell proliferation and cell death and is highly conserved in human and other mammalian organisms (Pan, 2007).

The first tumor suppressor of Hippo pathway, Warts (Wts), was identified in 1995 via genetic mosaic screening (Justice et al., 1995; Xu et al., 1995). It belongs to the Nuclear Dbf2/Dbf20-related (NDR) protein-Ser/Thr kinase. Loss of wts function results in overproliferation in both mosaic clones and homozygous mutants. At that time, Wts was like an “orphan” molecule, with the known identity of tumor suppressor but no interacting partners clarified or functioning mechanisms elucidated.

It was not until 7 years later that the ice of silence was broken. Other tumor suppressors that confer similar loss-of-function overgrowth phenotype and functionally interact with wts, were successively identified. Salvador (Sav), the WW domain-containing scaffold protein, promotes cell cycle exit and apoptosis (Kango-Singh et al., 2002; Tapon et al., 2002). Loss of sav delays cell cycle exit and inhibits apoptosis by inducing the expression of cell cycle regulator cyclin E and Drosophila inhibitor of apoptosis-1 (*diap1*), respectively. In the following year, Hippo (Hpo), a Ste-20 family protein-Ser/Thr kinase, was found to link Sav and Wts for growth control (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003). *hpo* mutation results in tissue overgrowth characterized by overproliferation and impaired apoptosis via increasing the level of Cyclin E and DIAP1. Upon phosphorylation within the kinase domain, Hpo can be activated and subsequently phosphorylates Wts kinase under the facilitation of Sav. Later on, Mob as tumor suppressor (Mats) was identified as a binding partner as well as activating subunit of Wts (Lai et al., 2005). Upon phosphorylation by Hpo, Mats is potentiated as a better activator of Wts kinase for growth inhibition (Wei et al., 2007). Together, Wts, Sav, Hpo and Mats form the core machinery of Hippo pathway as a kinase cascade to transduce upstream signal to downstream effectors.
Upstream to the core components, Merlin (Mer) and Expanded (Ex) are thought to relay the growth-inhibition signal to the Hippo pathway (Hamaratoglu et al., 2006). Mer and Ex are both FERM (4.1, Ezrin, Radixin, Moesin) domain cytoskeleton proteins and they are partially redundant in promoting cell cycle arrest and apoptosis. Pellock et al. showed that Ex and Mer have differential functions where Ex predominantly regulates cell proliferation whereas Mer regulates apoptosis (Pellock et al., 2007). Drosophila Ras association domain family protein (RASSF) homolog inhibits Hpo activity by competing with Sav for binding to Hpo (Polesello et al., 2006).

Fat (Ft) is a giant atypical protocadherin located on the apical junctions (Ma et al., 2003; Mahoney et al., 1991). Ft function is required for growth control (Bryant et al., 1988; Mahoney et al., 1991), planar cell polarity (PCP) (Casal et al., 2002; Saburi and McNeill, 2005) and proximal-distal patterning of appendages (Cho and Irvine, 2004). In response to extracellular ligand(s), Fat exerts its positive regulation on Hippo pathway for growth control. In terms of the Ft regulation of Hippo pathway, there are two mechanisms. The first one is the Ft-Ex-Hippo pathway, where Ft signals through Ex to regulate Hippo signaling (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006). The other one is Ft-Dachs-Wts, where Ft stabilizes Wts protein by inhibiting Dachs, an atypical myosin, in parallel to the activity regulation of Wts kinase by Hippo signaling (Cho et al., 2006; Feng and Irvine, 2007).

Yorkie (Yki) is a key downstream target of the Hippo pathway, negatively regulated by Wts kinase via direct phosphorylation (Huang et al., 2005). Overexpression of Yki causes severe overgrowth resembling the loss of function phenotypes of hpo, sav or wts. Requirement of yki for the overgrowth related to hpo, sav or wts mutation places yki downstream to these Hippo pathway components. Yki is a transcriptional coactivator which links the Hippo signal to target genes expression, such as cyclin E, diap1, ex, microRNA bantam, etc. (Nolo et al., 2006; Thompson and Cohen, 2006).

Transcriptional regulation of several target genes by Hippo pathway has been established. However, the identity of the transcription factor(s) that Yki utilizes to bind to DNA and therefore activates target gene expression was not known. It was not until early 2008 that the mystery of the sleeping beauty was uncovered. In chapter 3, I will present
the identification of the physical and functional partner of Yki, Scalloped (Sd), as a new component of Hippo pathway to mediate Yki-dependent tissue growth control (Zhao et al., 2008).

Recently, several new components of Hippo pathway have been identified, including Kibra (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010), Crumbs (Crb) (Grzeschik et al., 2010; Robinson et al., 2010), Lethal (2) giant larvae (Lgl), atypical protein kinase C (aPKC) (Grzeschik et al., 2010) and Drosophila Ajuba LIM (Lin11, Is11, Mec3) protein (dJub) (Das Thakur et al., 2010). Kibra is a cytoplasmic protein with two WW domains, functioning as tumor suppressor in Hippo signaling pathway (Kremerskothen et al., 2003). Similarly to but in a milder way than loss of core components of Hippo pathway, loss of kibra led to tissue overgrowth and upregulation of Hippo target genes expression. Kibra interacts physically and functionally with Mer and Ex to transduce growth regulatory signal to Hpo/Sav complex in apical domain of epithelial cells. Crb is a transmembrane protein with known functions in apicobasal polarity. It regulates Hippo pathway via its effects on Ex level and localization (Grzeschik et al., 2010; Robinson et al., 2010). Lgl and aPKC are both apicobasal cell polarity regulators. Grzeschik et al. reported that lgl depletion or aPKC overexpression led to tissue overgrowth via Hippo pathway through their regulation of Hpo and RASSF localization. dJub is another adherens junctions (AJs)-localized adaptor protein of Hippo pathway (Das Thakur et al., 2010). dJub negatively regulates Hippo signaling probably at the level of Wts.
1.5 Hippo Pathway in Mammalian Tumorigenesis

All of the known components of Hippo pathway are well conserved evolutionarily and many of them are implicated in the mammalian carcinogenesis. The *Drosophila* Hippo pathway components and their mammalian orthologs are listed in Table 1-1.
Table 1-1: Components of Hippo signaling pathway in *Drosophila* and Mammals.

<table>
<thead>
<tr>
<th>Drosophila melanogaster gene</th>
<th>Mammalian orthologs</th>
<th>Protein type</th>
<th>Role in mammalian cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>fat</td>
<td>FAT4</td>
<td>Protocadherin</td>
<td>Mutated in breast cancer</td>
</tr>
<tr>
<td>dachs (d)</td>
<td>Unknown</td>
<td>Atypical myosin</td>
<td>Unknown</td>
</tr>
<tr>
<td>crumbs (crb)</td>
<td>CRB</td>
<td>Transmembrane protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>expanded (ex)</td>
<td>EX1/Frmd6, EX2</td>
<td>FERM-domain protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>merlin (mer)</td>
<td>NF2/Merlin</td>
<td>FERM-domain protein</td>
<td>Mutated in familial cancer syndrome neurofibromatosis type 2</td>
</tr>
<tr>
<td>aPKC</td>
<td>PRKCI</td>
<td>Protein kinase C family Ser/Thr kinase</td>
<td>Required in multiple human cancer behaviors</td>
</tr>
<tr>
<td>lgl</td>
<td>LLGL1/2</td>
<td>Adaptor protein</td>
<td>Loss in wide range of tumors</td>
</tr>
<tr>
<td>kibra</td>
<td>KIBRA</td>
<td>WW domain scaffolding protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>djub</td>
<td>Ajuba</td>
<td>LIM domain adaptor protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>dRASSF</td>
<td>RASSF1</td>
<td>RA-domain protein</td>
<td>Hypermethylated in solid tumors</td>
</tr>
<tr>
<td>hippo (hpo)</td>
<td>MST1, MST2</td>
<td>Sterile-20 family Ser/Thr kinase</td>
<td>Hypermethylated in soft tissue sarcoma</td>
</tr>
<tr>
<td>salvador (sav)</td>
<td>WW45/SAV1</td>
<td>WW domain scaffolding protein</td>
<td>Mutated in renal cancer cell lines; Ablation in liver leads to hepatomas</td>
</tr>
<tr>
<td>warts (wts)</td>
<td>LATS1, LATS2</td>
<td>Nuclear Dbf2-related (NDR) family Ser/Thr kinase</td>
<td>LATS1deficient mice develop soft-tissue sarcomas and ovarian tumours; Hypermethylated in human breast cancer</td>
</tr>
<tr>
<td>mats</td>
<td>MATS1</td>
<td>Mob domain family NDR kinase cofactor</td>
<td>Mutated in human and mice cancer cell lines</td>
</tr>
<tr>
<td>yorkie (yki)</td>
<td>Yes-associated protein (YAP), TAZ</td>
<td>WW domain family transcription co-activator</td>
<td>YAP is amplified in human and mice tumors; Ectopic YAP promotes tumorigenesis in mice</td>
</tr>
<tr>
<td>scalloped (sd)</td>
<td>TEAD</td>
<td>Transcription factor</td>
<td>Unknown</td>
</tr>
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Neurofibromin 2 (NF2), the mammalian homolog of Merlin, is mutated in the familial cancer syndrome neurofibromatosis type 2 (McClatchey and Giovannini, 2005). WW45 gene is mutated in two renal cancer cell lines (Tapon et al., 2002), and the liver-specific ablation of WW45 causes hepatomas (Lee et al., 2010). Mice deficient of LATS1 (mammalian homologue of Wts) develop soft-tissue sarcomas and ovarian tumours (St John et al., 1999). Promoter hypermethylation and downregulation of LATS1/LATS2 or MST1/MST2 have been found in breast cancers and soft tissue sarcoma, respectively (Seidel et al., 2007; Takahashi et al., 2005). By sequencing the human and mouse tumor-derived expressed sequence tag (EST) of MATS cDNA sequences, our lab identified the mutations of MATS gene in human skin melanoma and mice mammary gland carcinoma (Lai et al., 2005). Moreover, the murine locus 9qA1, which contains only one known gene, Yes-associated protein (YAP), the mammalian homologue of *Drosophila* Yki, has been found to be highly amplified in mouse mammary tumors (Overholtzer et al., 2006). Its syntenic locus in human genome, 11q22, is also amplified in various human cancers (Hermsen et al., 2005; Lambros et al., 2005; Snijders et al., 2005). A recent report showed that Fat4 is inactivated in breast cancers and primary cancer cell lines (Qi et al., 2009).

The functional conservation of Hippo pathway components is supported by the observation that several mammalian homologues are able to rescue the phenotypes of *Drosophila* mutants, such as wts, hpo, mats or yki (Huang et al., 2005; Lai et al., 2005; Tao et al., 1999; Wu et al., 2003). The existence of an analogous signaling pathway in mammals is being gradually established, especially after YAP was shown to be negatively regulated by Hippo signaling via phosphorylation, cytoplasmic retention and consequently inhibition (Hao et al., 2008; Oka et al., 2008; Zhang et al., 2008a; Zhao et al., 2007). Mammalian Hippo signaling plays an important role in normal tissue growth and its dysfunction contributes to tumorigenesis. Actually it has been reported that gain of YAP or loss of Mst1/2 led to massive overgrowth and progression to tumorigenesis in mice (Camargo et al., 2007; Dong et al., 2007; Zhou et al., 2009). Ectopic YAP expands undifferentiated intestinal progenitor cells as well as neural progenitor cells (Camargo et
al., 2007; Cao et al., 2008). Cell culture studies revealed the involvement of Hippo pathway in contact inhibition (Morrison et al., 2001; Zhao et al., 2007).

Lgl and aPKC were newly identified as components of Hippo pathway in Drosophila. Mammalian LGL1/2 are well-known tumor suppressors due to the fact that deletion of Lgl is common in a variety of tumors, such as glioblastoma, colorectal carcinomas, melanoma, prostate, breast and lung cancers, etc (Humbert et al., 2008). On the other hand, human atypical protein kinase C iota (PRKCI) is an authentic oncogene (Fields and Regala, 2007). PRKCI is highly expressed in various human cancers and participates in multiple aspects of the transformed phenotype of human cancer cells, including growth, invasion and survival.

1.6 Regulatory Mechanisms of Hippo Signaling Pathway in Drosophila

The core kinase cassette of Hippo pathway, which consists of Wts, Hpo, Sav and Mats, is primarily regulated by phosphorylation and protein-protein interaction (Reddy and Irvine, 2008). Hpo kinase can be activated by intermolecular autophosphorylation in the kinase domain (Glantschnig et al., 2002; Lee and Yonehara, 2002). Activated Hpo then phosphorylates Sav and makes it a better scaffolding protein to bring Wts closer to Hpo for phosphorylation (Wu et al., 2003). Hpo also phosphorylates Mats, which promotes the Mats-Wts association and eventually activates Wts kinase activity (Wei et al., 2007). Active Wts thereby phosphorylates itself and other downstream targets, such as Yki (Figure 1-3).

The main downstream readout of Hippo kinase cassette is the phosphorylation and inhibition of Yki by Wts kinase and subsequently the blocking of target gene expression (Huang et al., 2005). Phosphorylation of Yki by Wts kinase facilitates its binding with 14-3-3 proteins and therefore sequesters Yki in cytoplasm in the inactive status (Basu et al., 2003; Dong et al., 2007; Zhao et al., 2007). When Hippo signaling is inactive, Yki, the transcription co-activator, enters nucleus and interacts with DNA-binding transcription factor, Sd, to induce Hippo target gene expression (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b; Zhao et al., 2008) (Figure 1-4). Hippo-
response enhancer of *diap1* gene has been identified, which is bound by Sd and mediates Sd-Yki-dependent gene regulation (Wu et al., 2008; Zhang et al., 2008b).

![Figure 1-3: Hippo signaling pathway “On” (active) state. Arrows represent activation effects. Bar-headed lines represent inhibition effects. Shaded proteins are negative components of Hippo pathway and are inactive when Hippo signaling is on.](image)

The upstream regulatory mechanism of Hippo pathway is more complicated and less known. Genetic analysis has placed *ex* and *mer* upstream of *hpo* as components of the same signaling pathway. The stronger severity of *ex* and *mer* double mutants than either single mutant suggests their functional redundancy in growth control (McCartney...
et al., 2000). Basouel et al. reported that Ex directly binds to Yki and retains Yki in the cytoplasm (Badouel et al., 2009a). Considering that Ex and Mer are able to influence Hpo and Wts phosphorylation (Hamaratoglu et al., 2006; Silva et al., 2006), the direct regulation of Yki by Ex is not likely to be the only way by which they are related to Hippo signaling. Actually a newly identified apical protein Kibra shows physical interactions with Mer, Ex and/or Hpo, Sav and Wts and is thought to activate Hippo pathway by nucleating the core Hpo kinase complex (Genevet et al., 2010; Yu et al., 2010). On the other hand, Hpo activity is inhibited by dRassf via its competition with Sav for binding to Hpo (Polesello et al., 2006). The question about how Hpo kinase cassette is activated is still elusive and worth investigating.

Regulation of Hippo signaling is interconnected to the previously known Fat signaling, which primarily comprises Fat, Dachs (D), Dachsous (Ds), Four-jointed (Fj) and Discs overgrown (Dco) (Reddy and Irvine, 2008). Fat regulates Hippo signaling by stabilizing Wts protein (Cho et al., 2006; Feng and Irvine, 2007) and affecting the protein level and/or subcellular localization of Ex (Bennett and Harvey, 2006; Feng and Irvine, 2007; Silva et al., 2006; Willecke et al., 2006). Both regulations are dependent on Dachs (Feng and Irvine, 2007). Another signal input through Ex is from a transmembrane protein, Crb, which regulates the protein level and/or localization of Ex (Grzeschik et al., 2010; Robinson et al., 2010).
In a word, regulation of Hippo pathway mainly includes kinase cascade phosphorylation, protein-protein interactions mediated by scaffold proteins, such as Kibra, Mer, Ex, Sav and Mats, and shuffling of proteins between different subcellular compartments, such as the cytoplasm-nucleus transportation of Yki and membrane targeting and detachment of Ex, etc. In addition, ex and kibra are both transcriptional targets of Hippo pathway, acting as negative feedback probably to maintain Hippo signaling in a steady state (Genevet et al., 2010; Hamaratoglu et al., 2006). The “On”
(active) or “Off” (inactive) state of Hippo signaling pathway is shown in Figure 1-3 and Figure 1-4, respectively.

1.7 Regulation of Mammalian Hippo Signaling Pathway

The core components of *Drosophila* Hippo signaling pathway are well conserved in mammals, so is the regulatory mechanism of the Hippo kinase cascade (Badouel et al., 2009b). MST1/2 (Hippo homologs) autophosphorylate and phosphorylate WW45 (Sav homolog), which facilitates the phosphorylation and activation of LATS1/2 (Wts homologs) by MST proteins (Callus et al., 2006; Chan et al., 2005; Praskova et al., 2004). MST proteins also phosphorylate MATS (Mats homolog) (Praskova et al., 2008). MST-phosphorylated MATS has higher affinity for LATS1 and functions as a better activating subunit (Hergovich et al., 2006; Praskova et al., 2008).

Little is known about the regulatory mechanisms upstream to the core kinase complex, although the plasma membrane has been shown to be an important platform for the activation of Hippo signaling. Human LATS1 is activated by human MOB1 (MATS) at the membrane (Hergovich et al., 2005; Hergovich et al., 2006). Moreover, RASSF1 directs MST to plasma membrane for activation (Praskova et al., 2004). NF2 (Mer), the well known tumor suppressor in mammalian nervous system, also needs membrane association for activation (Okada et al., 2007).

Downstream to the kinase complex, active LATS1 phosphorylates YAP and TAZ (Yki homologs), two transcriptional coactivators which have been shown to be important for regulating target gene expression of mammalian Hippo pathway (Hao et al., 2008; Kanai et al., 2000; Lei et al., 2008; Sudol, 1994; Zhang et al., 2008a; Zhao et al., 2007). Similarly to the regulation of Yki by Wts in *Drosophila*, phosphorylated YAP/TAZ is retained in the cytoplasm by association with 14-3-3 protein whereas the dephosphorylated YAP/TAZ enters nucleus and binds to TEAD transcription factors (Sd homologs) to activate target gene transcription. The details are shown below.
1.8 Identification of TEAD as YAP-interacting Transcription Factor

YAP was originally identified as c-Yes-associated protein (Sudol, 1994). YAP is the proline-rich phosphoprotein containing one or two WW domains and a C-terminal transcription activation domain. There are two different splicing isoforms in human, YAP1 and YAP2. YAP1 is the short form lack of the second WW domain.

The transcriptional co-activator function of YAP was firstly demonstrated by Yagi et al., showing that fusion of C-terminal region of YAP to Gal4 DNA-binding domain strongly induces transactivation of reporter gene (Yagi et al., 1999). They identified PEBP2α (polyomavirus enhancer binding protein 2, α subunit) as YAP-cooperating transcription factor via PPXY motif-WW domain interaction. Later on, another two PPXY motif-containing transcription factors, p73 and ErbB-4, were also reported to mediate YAP-dependent gene transcription (Komuro et al., 2003; Strano et al., 2001).

YAP also binds to transcription factors, such as TEAD proteins, via other mechanisms rather than PPXY-WW domain interaction (Vassilev et al., 2001). TEAD transcription factors belong to DNA-binding TEA/ATTS domain family and there are four closely related TEAD proteins in mammals, TEAD1-4 (Campbell et al., 1992). YAP was identified as TEAD-binding protein by affinity chromatography (Vassilev et al., 2001). A novel N-terminal TEAD-binding domain (TBD) of YAP is required for its binding to the C-terminal YAP-binding domain (YBD) of TEAD. The C-terminal acidic activation domain of YAP is required for TEAD-dependent transcription activation. Zhao et al. independently identified TEAD as the strongest functional partner of YAP by a transcription reporter assay (Zhao et al., 2008). TEAD was shown to be essential for YAP-induced gene expression, overgrowth, epithelial-mesenchymal transition (EMT) and oncogenic transformation in MCF10A cells (human mammary epithelial cell line).

1.9 Abbreviations and Acronyms Used in the Thesis
Table 1-2: Summary of Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviations or Acronyms</th>
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<tr>
<td>AJs</td>
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<td>YAP</td>
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<td>Yki</td>
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Chapter 2

Materials and Methods

2.1 DIG Labeling of dAkt1 RNA

cDNA clone of dAkt1 in pOT2 vector (SD10374) was purchased from Drosophila Genomics Resource Center. Recovery of DNA from Whatman FTA discs was performed according to the DGRC Standard Operating Procedure. DH5α competent cells were used to amplify DNA construct in LB broth (10g tryptone, 5g yeast extract, 10g NaCl per 1L medium) containing 68ug/ml chloramphenicol. Extraction and purification of plasmid DNA was done using Wizard Plus SV Minipreps DNA Purification System (Promega, Cat. No. A1460). Dissolve DNA in nuclease free water and the final DNA concentration should be around 500ng/ul. T7 and Sp6 primers (provided by the Genomics Core Facility) were used to sequence the DNA.

About 10ug template DNA was linearized by EcoRI digestion overnight at 37°C (or XhoI digestion for generating sense riboprobe as negative control). Cutting efficiency was checked by gel electrophoresis. Linearized DNA was phenol/chloroform purified and redissolved in 10ul DEPC- ddH2O. About 1ug of this DNA was in vitro transcribed. Sp6 polymerase (or T7 polymerase for negative control probe) was used to generate DIG-labeled RNA as instructed by manufacturer’s protocol (DIG RNA labeling Kit (SP6/T7), Roche 11175025910). A mini native gel was run for 30 min to check the presence of RNA products. RNA products were then ethanol precipitated and dissolved in 100ul DEPC- ddH2O.
2.2 Detection of mRNA and GFP Protein Simultaneously in Drosophila Wing Imaginal Discs via Whole-mount in situ Hybridization and Immunostaining

Wing imaginal discs from the late third instar larvae were dissected in PBS and fixed in 4% paraformaldehyde, PBS, 0.5% Triton X-100 on ice for 15-20 min while continuing to dissect more discs. Additional 20 min fixation was performed at room temperature using the same fixative as above. Following fixation, discs were washed 5 min x 3 times in PTw (0.1% Tween-20 in PBS) at RT on a shaker.

The discs were then washed for 10 min with 1:1 PTw:Hybridization buffer (50% (v/v) formamide, 5x saline sodium citrate (SSC, pH 5.0), 50ug/ml yeast tRNA, 50ug/ml heparin, 0.1% (v/v) Tween-20). Next, the discs were incubated in hybridization buffer for 10 min. The discs were then transferred from glass dishes to PCR tubes and were prehybridized for 1 hour in PCR machine at 60°C. During this time, 2ul DIG-labeled riboprobe was boiled for 2-5 min at 100°C, and then cooled shortly on ice. 200ul hybridization buffer was added and vortexed briefly. The volume of riboprobe and hybridization buffer should be scaled up if more than one hybridization was carried out. After the 1 hour incubation, most of the prehybridization solution was removed and the denatured 200ul riboprobe solution was added. The samples were then incubated at 60°C overnight without rocking.

Next day, the following solutions were prepared and pre-warmed at 60°C: hybridization buffer, hyb buffer/PTw (4/1), hyb buffer/PTw (3/2), hyb buffer/PTw (2/3), hyb buffer/PTw (1/4), PTw, PTw. The discs were washed for 20 min at 60°C in each of these solutions in the order as listed. The discs were then blocked for 20 min x 2 times at RT in PBTN (1xPBS, 0.1% BSA, 0.2% Triton X-100, 5% NGS). Anti-DIG-Rhodamine antibody stock solution was diluted 1:20 in blocking buffer (PBTN) and centrifuged for 5 min at 10,000 rpm prior to use. Blocking buffer was removed and 100ul of the diluted Ab solution was added to each vial. The samples were incubated for 2 hours at RT. After being washed 20 min x 4 times in 0.2% PBST (0.2% Triton X-100 in PBS), the discs were incubated in rabbit anti-GFP Ab solution (1:100 in blocking buffer) overnight at 4°C. In the following day, the discs were washed with 0.2% PBST for 1 hour (5 min x 3 times and then 15 min x 3 times) and then incubated in appropriate secondary Ab
solution at RT for 2 hours (e.g. anti-rabbit Alexa Fluor 488 1:200-1:500 diluted in blocking buffer). The discs were then washed with 0.3% PBST (0.3% Triton X-100 in PBS) for 1 hour (5 min x 3 times and then 15 min x 3 times). The discs were rinsed with PBS for 5 min and then transferred to glass dishes with 50% glycerol in PBS for 1-2 hours at RT. 70% glycerol in PBS was next used to equilibrate the discs for several hours at 4°C. The discs were then mounted onto slides with parafilm as the spacing between slide and cover slip due to the fragility of samples. Images were collected with FluoView FV300 confocal microscope.

2.3 Preparation of Fly Food

Weigh and mix the following amount of dry ingredients for 25 liter food: 237.5 g agar, 975 g cornmeal, and 662.5 g baker’s yeast. Cook 32 liter of water till the steam starts to show. Extra amount of water was proved important to compensate the loss from evaporation and to make the fly food with appropriate hardness. Gently pour the dry mixture into the warm water while keep stirring. Cook 15-30 min or till cornmeal particles are dissolved. Add 1612.5 ml molasses and continue to cook for another 5-10 min. Keep eyes on the food mixture during cooking since it is very easy to boil over. Let the food mixture cool off to 58°C, which takes about 1 hr. Prepare 160 ml propionic acid and 480 ml tegosept solution (48 g methyl p-hydrobenzoate in 480 ml 95% ethanol). Once the food temperature reaches 58°C, add propionic acid and tegosept solution and mix well. The food is now ready for dispensing. Use 7 ml for small vial, 10 ml for large vial and 30 ml for bottle.

2.4 Preparation of CaCl₂ Chemical Competent Cells

CaCl₂ chemical competent cells (e.g. DH5α, XL10) were prepared as described below.
One single colony was inoculated in 2-3ml LB medium without antibiotics and incubated overnight at 37°C with shake. Overnight culture was added into 480ml LB medium containing 10mM MgCl$_2$ and continued to grow at 37°C with shake until the A600 reached 0.5-0.7 (about 3 hours). Cell culture was then divided into 2 sterile Beckman bottles. Bottles were balanced and cooled on ice.

Cells were centrifuged at 3000g for 5 min at 4°C and the supernatant was poured. 60ml of cold, sterile 0.1M MgCl$_2$ solution was added into each bottle. The bottles were balanced, the cells were gently resuspended using 10ml pipette, and then incubated on ice for 20 min.

Cells were again centrifuged at 3000g for 5 min at 4°C and the supernatant was poured. 6ml of cold, sterile 0.1M CaCl$_2$ solution containing 19% glycerol was added into each bottle. Cells were gently resuspended, combined into one bottle, and then incubated on ice for 40 min.

Cells were divided into 100ul aliquots per tube and quickly frozen in liquid nitrogen. Cell aliquots were stored at -80°C.

2.5 Microinjection for Transgenic Flies

Transgenic flies were generated based on P-element transformation (Rubin and Spradling, 1982). First of all, gene of interest was subcloned into pUAST vector, which contains five tandemly arrayed GAL4 binding sites for the regulation of gene expression via GAL4-UAS system. The vector also includes P element ends (P3' and P5') for transposition purpose and a white gene as a marker for successful incorporation of transgene (Brand and Perrimon, 1993).

Plasmid DNA was purified with Wizard Plus SV Minipreps DNA Purification System (Promega) and dissolved in ddH$_2$O for a final concentration of 300-400ng/ul. 1ul green food dye was added into 10ul DNA solution and it was centrifuged at 10,000 rpm for 10 min. The supernatant was collected as the working solution. $w^+; Dr / TMS$Δ2-3
Sb\(^{-}\) blastoderm embryos provide constant source of transposase and were used as transgene recipient. Fresh eggs were collected every 30 min and washed briefly with distilled water. The moist eggs were lined up in the same direction on glass cover slips and were left in 20\(^\circ\)C room to air dry. Right after the visible moist on the cover slip evaporated, the eggs were covered with halocarbon oil mix (9 parts of 700 halocarbon oil and 1 part of 27 oil) and subject to microinjection. The injected embryos were left in Amp\(^{+}\) LB plates and allowed to develop in 20\(^\circ\)C room overnight. Hatched larvae were carefully transferred to food vial and incubated at 25\(^\circ\)C. G\(_0\) adult flies were mated with \(w^{-}\) flies and the red-eyed progenies were the ones with successful incorporation of transgene and the marker allele (\(w^{+}\)) into the recipient genome. These red-eyed G1 adult flies were then crossed with \(w^{-};\ \text{Adv/SM1; Sb/TM6}\) serially to identify in which chromosome the transgene had been inserted and to balance the transgenic flies.

2.6 Drosophila Schneider 2 (S2) Cells Culture

Drosophila S2 cell stock was a gift from Dr. Susan Abmayr. S2 cells were kept in Schneider’s Drosophila Medium containing 10\% heat-inactivated FBS, 50 units penicillin and 50 μg streptomycin per ml medium. Cells were incubated at 25\(^\circ\)C without CO\(_2\).

When cell clumps were obviously formed in culture medium, the density was about 1x10\(^7\) cells/ml and the cells were split into new medium at 1:5 dilution. To do so, tap the flask several times to dispatch the cells and pipette up and down the medium to wash the cells out of the surface. The cells were then diluted at 1:5 in fresh medium.

2.7 Transient Transfection of Drosophila S2 Cells

Cell pellets were resuspended in Schneider’s medium free of serum and antibiotics. 1.5ml cell medium was plated in one well of 6-well plate and incubated at
25°C for at least 1 hour. During the incubation time, 10µl Cellfectin Reagent (Invitrogen, Cat. No. 10362-010) was added to 100µl Schneider’s medium free of serum and antibiotics, and 1µg DNA (pAc5.1-yki-V5 or pAc5.1-yki<sup>S97A</sup>-V5) was added separately into another tube of 100µl Schneider’s medium free of serum and antibiotics. Two tubes of medium were combined and mixed well and then incubated at RT for 15-20 min. 200µl medium complex was added into the cell and gently swirled for even distribution. Cells were incubated at 25°C without CO₂ and were harvested 36-48 hours later.

2.8 Total Protein Harvest of *Drosophila S2* Cells

All steps were performed at 4°C. Cells were transferred to 15ml tube and centrifuged at 1000 rpm for 4 min. The cell pellet was washed with cold PBS twice. 200µl lysis buffer was added and the sample was moved to 1.5ml centrifuge tube. The cells were vortexed 30 seconds and left on ice for 25 min with another 30 seconds vortex in the middle of incubation. The cells were then centrifuged at 15,000 rpm at 4°C for 10 min. The supernatant was collected and stored at -20°C.

Basic components of lysis buffer: 50mM Tris-HCl (pH 7.4), 150mM NaCl, 2mM EDTA (pH 8.0), 1% Triton X-100, 10% glycerol. The following components were added freshly right before use to make the final lysis buffer: 2mM DTT, 1mM PMSF, 10mM NaF, 2mM Na<sub>3</sub>VO<sub>4</sub>, 60mM glycerol 2-phosphate and 1:500 protease inhibitor cocktail (Sigma).

2.9 Expression of GST-Tagged Sd Protein in BL21

cDNA clone of *sd* in pGEX 5X-1 was a gift from Dr. Sean Carroll. GST-tagged Sd protein was expressed in BL21 cells. 100µl BL21 competent cells were transformed with 100-150ng pGEX-sd plasmid DNA and plated on Amp<sup>+</sup> LB (LBA) plate to grow overnight at 37°C. One big colony was picked up and inoculated in 20ml LBA medium, and then was incubated overnight at 37°C with shake. 10ml of the overnight culture was
transferred into 100ml LBA medium and raised for about 1 hour at 37°C with shake. When OD600 reached 0.5-1.0, 70ul of 1M IPTG was added to induce the protein expression. The culture was incubated at 37°C for another 3-4 hours with shake. The cultures were next transferred to Beckman bottles and centrifuged at 5000rpm at 4°C for 15 min. The supernatant was discarded and the pellet could be stored at -80°C.

2.10 Purification of GST-Tagged Sd Protein

Bacteria pellet from 100ml culture was thawed and resuspended in 5ml lysis buffer (100mM NaCl, 100mM Tris pH 8.0, 50mM EDTA, 2% Triton X-100), 1ml freshly made lysozyme solution (10mg/ml in lysis buffer), and 6ul PMSF (100mM, ~10mg/ml). The resuspended solution was transferred to 50ml Beckman tube and left on ice for 30 min. The tube was then centrifuged at 13,000rpm for 30 min at 4°C. During this spinning time, a cut tip P1000 was used to take 200ul 50% glutathione beads slurry to 15 ml VWR centrifuge tube and left on ice. When the beads settle down to the bottom, some of the supernatant was removed to decrease the dilution. After spinning, the supernatant of lysed bacteria was transferred to the beads slurry and rotated at 4°C for 30 min to overnight to allow the protein binding. The protein solution was then centrifuged at 1000rpm at 4°C for 30 seconds. The supernatant was removed and the beads pellet was washed three times with 1% Triton X-100 PBS. The beads with small amount of wash solution were transferred to 1.5ml tube with cut tip.

If the protein was going to be used in the near future for pull-down assay, 1% Triton X-100 PBS could be added to the tube till 1ml, and 300ul of the solution could be saved and left in 4°C for use. The rest of the solution was centrifuged at 1000rpm for 30 seconds and the supernatant was removed. 100ul of the elution buffer with reduced glutathione (30 mg reduced glutathione in 10ml 50mM Tris pH 8.0) was added and rotated at room temperature for 10 min. The solution was then centrifuged at 1000rpm for 30 seconds and the supernatant was collected. Protein concentration was determined at wave length 595nm of Spectronic GENESYS 5, using Bio-Rad Protein Assay (Cat. No. 500-0006).
2.11 *in vitro* Pull Down Assay

GST-tagged Sd protein was used as bait and immobilized on glutathione sepharose beads. The total extracts of S2 cells overexpressing Yki or Yki$^{S97A}$ were used as prey to examine the physical interactions of Sd&Yki and Sd&Yki$^{S97A}$. 25ul of the GST-Sd binding beads solution was combined with 50ul total S2 cell lysate (both protein solutions were prepared as described earlier) and 1% Triton X-100 PBS was added to make a final volume of 400ul in a 1.5ml tube. The solution was incubated at 4°C for 2 hours to overnight with rocking. After binding, the protein solution was centrifuged at 1000rpm for 30 seconds and the beads were washed three times with 1% Triton X-100 PBS. 6 X SDS loading buffer was added and the beads were boiled 5 min at 100°C. The supernatant was collected for Western Blot.

<table>
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<th>Table 2-1 Components of 6X SDS Loading Buffer</th>
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<td>1.5M Tris-HCl, pH 6.8</td>
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<tr>
<td>SDS</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>H$_2$O</td>
</tr>
</tbody>
</table>

2.12 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Before performing the western blot, proteins were first separated by SDS-PAGE. Resolving gel (7.5%) was prepared according to the recipe below.
Table 2-2 Components of 7.5% Resolving Gel

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>10ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O</td>
<td>4.85ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td>2.5ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bisacrylamide Mix</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100ul</td>
</tr>
<tr>
<td>10% Ammonium Persulfate (freshly made)</td>
<td>50ul</td>
</tr>
<tr>
<td>TEMED</td>
<td>5ul</td>
</tr>
</tbody>
</table>

Resolving gel was poured into assembled Bio-Rad Mini-PROTEAN II apparatus till about ¾ height of the small glass and a thin layer of isobutanol was applied on top of resolving gel to seal and flat the top lining. While waiting for the polymerization to occur (about 20-30 min), the stacking gel was made according to the recipe below.

Table 2-3 Components of Stacking Gel

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O</td>
<td>3.05ml</td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>1.25ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bisacrylamide Mix</td>
<td>0.65ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50ul</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>25ul</td>
</tr>
<tr>
<td>TEMED</td>
<td>5ul</td>
</tr>
</tbody>
</table>

Isobutanol was removed and the stacking gel was added. Comb was gently inserted, preventing the formation of any air bubbles between the comb and gel. Protein
samples were combined with 6X SDS loading buffer and boiled for 5 min at 100°C. The supernatant was collected to load the SDS gel. Gel was run at 160V for about 1 hour till the dye reached the bottom of gel. Every 1L of 10X running buffer contained 30g Tris, 144 g Glycine, and 10g SDS.

To estimate the quantity of target proteins, the gel could be stained with Coomassie Brilliant Blue by merging in staining solution (1% Coomassie Brilliant Blue R-250, 40% Methanol, 10% Glacial Acetic Acid) with shake for 30 min to several hours. Destain solution (40% Methanol, 10% Glacial Acetic Acid) was used to destain the gel overnight with shaking.

2.13 Western Blot

For western blot, proteins on the gel were transferred to nitrocellulose membrane electrophoretically at 100V for 1 hour (or 30V overnight in the cold room). 25mM Tris (pH 8.3), 192mM Glycine, and 20% v/v Methanol was used as transfer buffer. Nitrocellulose membrane with proteins bound was then incubated in blocking buffer (5% nonfat dry milk in PBST (0.1% Tween-20 in PBS)) for 1 hour at room temperature with gentle agitation. After blocking, the membrane was transferred to a plastic bag followed by the sealing of three margins, and then the diluted primary antibody in blocking buffer was added. The fourth margin was sealed too and the membrane was incubated overnight at 4°C with rocking. The primary antibody solution was collected and could be recycled several times. The membrane was washed 5 min twice in PBST with gentle agitation. The membrane was then incubated in HRP-conjugated secondary antibody solution diluted in blocking buffer in a sealed plastic bag and was agitated for at least 1 hour at room temperature. The membrane was again washed 5 min twice in PBST with gentle agitation. ECL+ Plus detection reagents (Amersham) were used to perform the detection reaction. Membrane was incubated in the mixture of solution A and B (40:1) for 5 min in the dark and then exposed to the autoradiography film for 30 seconds to 10 min.

If necessary, the membrane could be stripped and re-probed with other antibodies. The membrane was first washed three times with PBS and was then incubated in
stripping buffer (2% SDS, 62.5mM Tris-HCl pH 6.8, and 100mm β-mercaptoethanol) for 30 min at 50°C with gentle agitation. After stripping, the membrane was washed three times with PBS and re-blocked in blocking buffer for 1 hour. The membrane was then ready for antibody blotting.

2.14 Preparation of Fly Endogenous Protein for Western Blot

Late third instar larvae of flies were dissected in PBS and the tissue of interest (e.g. eye imaginal discs, wing imaginal discs, etc.) was left in PBS on ice. The tissue was transferred to 1.5ml tube and quickly centrifuged. The supernatant was removed and 20ul PBST (0.1% Tween-20 in PBS) was added. The tissue was quickly frozen in liquid nitrogen and then was homogenized with plastic pestle. 6X SDS loading buffer was added and the tissue was boiled for 5 min at 100°C. Tissue sample was centrifuged at 13,000rpm for 10 min at room temperature and the supernatant was applied to SDS-PAGE followed by western blot.

2.15 Genetic Interaction of yki and sd

To test the genetic interaction between yki and sd, three sets of experiments were performed. The adult fly eye images were taken by Scanning Electron Microscopy (SEM).

The first one was to combine the overexpression of yki with loss of one allele of sd. Males of w; GMR-Gal4, UAS-yki-V5/S. T. were crossed with females of y w; sd^{12} FRT18A/FM7a. w; GMR-Gal4/UAS-yki-V5; +/- and y w; sd^{12} FRT18A/+ were used as controls.

The second one was to combine the overexpression of sd with loss of one allele of yki. Males of w; GMR-Gal4; UAS-sd / S.T. were crossed with females of w; FRT42D yki^{B5} /S. T.. w; GMR-Gal4/++; UAS-sd/+ and w; FRT42D yki^{B5} /+ were used as controls.
The third one was to coexpress yki and sd. The adult eye phenotypes of \( w; GMR-Gal4/UAS-yki-V5; UAS-sd/+ \) flies were compared with the adult eye phenotypes of \( w; GMR-Gal4/+; UAS-sd/+ \) and \( w; GMR-Gal4/UAS-yki-V5; +/+ \) flies.

### 2.16 Scanning Electron Microscope

Adult flies were dehydrated in a series of ethanol concentrations. To do so, the flies were soaked for at least 2 hours in 25%, 50% and 75% ethanol subsequently, and then were left in 100% ethanol overnight. The samples were critical point dried using BAL-TEC CPD030, and then the completely dried samples were mounted onto standard metal stub with double sided carbon tape and were sputter coated with gold and palladium using Bal-TEC SCD-050 Sputter Coater. Images were taken using JSM 5400 Scanning Electron Microscope.

### 2.17 FLPout System for Clonal Overexpression of Transgenes

To induce the clonal overexpression of transgene, males carrying the transgene downstream of UAS were crossed with females of \( w, hsFLP; act>w^+>Gal4; UAS-GFP/TM6B \). For the control experiment, 176 males were mated with \( w, hsFLP; act>w^+>Gal4; UAS-GFP/TM6B \). Crosses were performed at 20°C. The parental flies were removed 1 day after egg laying (AEL) and the eggs were heat shocked for 1 hour at 31°C 4 days AEL. The eggs continued to grow at 20°C and wing imaginal discs of late 3\textsuperscript{rd} instar larvae were used for experimental analysis. The clones overexpressing transgenes were positively marked by GFP fluorescence.
2.18 Clonal Analysis by Positive Labeling

To generate GFP positively labeled *mats* mutant clones, males of *w*; *FRT82B mats*\textsuperscript{235}/TM6B were mated with females of *yw*, *hsFLP*, *UAS-GFP*, *tub-Gal4*, *FRT82B tub-Gal80/TM6B* (gift from Dr. Norbert Perrimon). For the control experiment, males of *w*; *FRT82B P[w\textsuperscript{+}]/S.T. were mated with females of *yw*, *hsFLP*, *UAS-GFP*, *tub-Gal4*, *FRT82B tub-Gal80/TM6B*. Flies were incubated at 25°C. The parental flies were removed 1 day after egg laying (AEL) and the eggs were heat shocked for 2 hours at 37°C 2 days AEL. Late 3\textsuperscript{rd} instar larvae were dissected and the wing imaginal discs were fixed and used for experimental analysis.

Similarly, to generate GFP positively labeled *hpo* mutant clones, males of *w*; *FRT42D hpo\textsuperscript{BF33}/SM6, TM6B (S.T.) were crossed with females of *hsFLP122, UAS-GFP*, *FRT42D tub-Gal80; tub-Gal4/TM6B*. For control crosses, *w*; *FRT42D P[w\textsuperscript{+}]/S.T. were crossed with females of *hsFLP122, UAS-GFP*, *FRT42D tub-Gal80; tub-Gal4/TM6B*. The heat shock condition was the same as generating *mats* mutant clones.

2.19 Epistasis Analysis of *pi3k* and *mats*

UAS-pi3kRNAi line was ordered from Vienna Drosophila RNAi Center (VDRC). MARCM analysis was performed to examine the epistatic relationship of *pi3k* and *mats* genes. Males of *w*; *UAS-pi3kRNAi*, *FRT82B mats*\textsuperscript{235}/S.T. were mated with females of *yw*, *hsFLP*, *UAS-GFP*, *tub-Gal4*, *FRT82B tub-Gal80/TM6B*. Overexpression of pi3kRNAi in wild type clones was generated by crossing males of *w*; *UAS-pi3kRNAi; FRT82B P[w\textsuperscript{+}]/S.T. with females of *yw*, *hsFLP*, *UAS-GFP*, *tub-Gal4*, *FRT82B tub-Gal80/TM6B*. *mats* mutant clones and wild type control clones were obtained by crossing males of *w*; *FRT82B mats*\textsuperscript{235}/TM6B or males of *w*; *FRT82B P[w\textsuperscript{+}]/S.T. with females of *yw*, *hsFLP*, *UAS-GFP*, *tub-Gal4*, *FRT82B tub-Gal80/TM6B* with females of *yw*, *hsFLP*, *UAS-GFP*, *tub-Gal4*, *FRT82B tub-Gal80/TM6B*.

Flies were incubated at 25°C. The parental flies were removed 1 day after egg laying (AEL) and the eggs were heat shocked for 2 hours at 37°C 2 days AEL. Late 3\textsuperscript{rd}
instar larvae were dissected and the wing imaginal discs were fixed and used for experimental analysis. Immunostaining with antibodies against p-Akt, or p-4E_BP1 was performed as described. GFP signal labeling the clone region and Alexa-conjugated secondary antibodies were visualized with FV300 confocal microscopy.

2.20 Epistasis Analysis of akt and mats

UAS-aktRNAi line was also ordered from VDRC. Similarly to the study of epistatic relationship of pi3k and mats genes, males of w¹;UAS-aktRNAi; FRT82B mats235/S.T. were crossed with females of y¹w¹, hsFLP, UAS-GFP; tub-Gal4, FRT82B tub-Gal80/TM6B. To generate control clones, females of y¹w¹, hsFLP, UAS-GFP; tub-Gal4, FRT82B tub-Gal80/TM6B were crossed with males of w¹; UAS-aktRNAi; FRT82B P[w¹]/S.T., males of w¹; FRT82B mats235/TM6B or males of w¹; FRT82B P[w¹].

Flies were incubated at 25°C. The parental flies were removed 1 day after egg laying (AEL) and the eggs were heat shocked for 2 hours at 37°C 2 days AEL. Late 3rd instar larvae were dissected and the wing imaginal discs were fixed and used for experimental analysis. Immunostaining with Akt or p-4E_BP1 antibodies was carried out and the images were taken via FV300 confocal microscopy.

2.21 Epistasis Analysis of yki and mats

UAS-ykiRNAi line was obtained from VDRC. Double mutant clones were generated by MARCM as well. Males of w¹;UAS-ykiRNAi; FRT82B mats235/S.T. were crossed with females of y¹w¹, hsFLP, UAS-GFP; tub-Gal4, FRT82B tub-Gal80/TM6B. For control clones, females of y¹w¹, hsFLP, UAS-GFP; tub-Gal4, FRT82B tub-Gal80/TM6B were crossed with males of w¹; UAS-ykiRNAi; FRT82B P[w¹]/S.T., males of w¹; FRT82B mats235/TM6B or males of w¹; FRT82B P[w¹].

Flies were incubated at 25°C. The parental flies were removed 1 day after egg laying (AEL) and the eggs were immediately heat shocked for 2 hours at 37°C after
removing the adults. Late 3\textsuperscript{rd} instar larvae were dissected and the wing imaginal discs were fixed and used for experimental analysis. Immunostaining against Akt protein was performed and the images were taken via FV300 confocal microscopy.

2.22 Immunostaining of Mid-Pupal Eye Discs

Immunofluorescent staining of mid-pupal eye discs was performed with mouse anti-Discs large (Dlg) (DSHB, 1:300) as primary antibody and Alexa Fluor 488 (Molecular Probes, 1:300) as secondary antibody. Images were collected with FV300 confocal microscopy.

2.23 Adult Wing Size Measurement and Statistical Analysis

Male flies carrying UAS-transgenes were crossed carrying driven by C5-Gal4. Adult male fly wings overexpressing UAS-transgenes driven by C5-Gal4 were collected and mounted onto the glass slides. Wing images were taken under dissecting microscope via SPOT Basic Imaging software. The wing area in pixels was measured using Image J or Photoshop CS3. The wing sizes were normalized to the control wings. Statistical analysis was performed using t-Test (P=0.05).
Chapter 3

Sd Interacts with Yki to Control Tissue Growth

3.1 Introduction

Hippo signaling pathway is a novel tumor suppressor pathway identified in *Drosophila* and all known components are conserved in mammals (Edgar, 2006; Harvey and Tapon, 2007; Pan, 2007). Yki functions as transcriptional co-activator to regulate target gene expression of Hippo pathway, such as *cyclin E* and *diap1* (Huang et al., 2005). The gene regulation activity of Yki is inhibited via direct phosphorylation by Wts kinase (Dong et al., 2007). Yki is lack of DNA-binding domain and its regulation of target gene expression has to be mediated by some DNA-binding transcription factor(s).

YAP is the mammalian homolog of *Drosophila* Yki. YAP functions as transcriptional co-activator by collaborating with several transcriptional factors, such as the four transcriptional enhancer factors (TEFs), the p53 family member p73, and Run transcription factors (Strano et al., 2001; Vassilev et al., 2001; Yagi et al., 1999). TEFs proteins share the TEA DNA-binding domain of 66-68 amino acids (Campbell et al., 1992). They play important role in cardiogenesis, myogenesis, organogenesis, and neural development (Campbell et al., 1992; Jacquemin et al., 1997; Xiao et al., 1991). The human TEF-1 was identified in Hela cells, showing the ability to bind to the GT- II C and Sph (I+ II) enhansons in the simian virus 40 (SV40) enhancer (Davidson et al., 1988). In addition to TEF-1 (renamed as TEAD1), human genome contains three more TEA domain proteins, TEF-4 (TEAD2), TEF-5 (TEAD3) and TEF-3 (TEAD4), and all of them show binding potential with YAP (Jacquemin et al., 1996; Jacquemin et al., 1997). The TEA domain proteins are also found in other eukaryotes, such as the yeast TEC1, C. elegans EGL-44, and Aspergillus AbaA (Laloux et al., 1990; Sewall et al., 1990; Wu et al., 2001).
Sd is the only TEA domain protein in *Drosophila*. Previous studies have shown that Sd functions by partnering with Vestigial (Vg), the wing-specific transcriptional cofactor, to regulate wing-specific gene expression, promote wing morphogenesis and induce cell proliferation and cell survival of the dorsal-ventral boundary of wing imaginal disc (Delanoue et al., 2004; Paumard-Rigal et al., 1998; Simmonds et al., 1998). However, Vg is unlikely to be the only partner for Sd. The expression of vg is limited to the wing and haltere imaginal discs whereas sd is generally expressed in multiple tissues and required for their development, such as the embryonic nervous system, the larva wing, eye-antennal and leg imaginal discs (Campbell et al., 1992; Campbell et al., 1991; Williams et al., 1991). This is suggestive of additional partner(s) of Sd acting outside the wing to regulate gene expression, cell differentiation, cell proliferation and/or cell survival.

Kun-Liang Guan’s group identified TEAD as the most potent target to mediate YAP-dependent gene expression (Zhao et al., 2008). In addition, they showed that TEAD-binding is required for YAP-induced cell growth, anchorage-independent growth, and epithelial-mesenchymal transition (EMT). The interaction between Yki and Sd is supported by yeast two-hybrid screen (Giot et al., 2003). My work described in this chapter demonstrated that Sd functions as the partner of Yki to coordinately promote tissue growth in *Drosophila*.

### 3.2 Yki and Sd Physically Interact in vitro

Zhao *et al.* identified TEAD as the important transcription factor of YAP and found that the point mutation of Ser 94 to Ala of YAP blocks its interaction with TEAD proteins (Zhao et al., 2008). We wanted to examine whether Yki and Sd also interact with each other and whether Ser 97 of Yki (corresponding to Ser 94 of YAP) is critical for Sd-binding.

The *in vitro* pull-down assay was performed. GST-Sd (a gift from Sean Carroll) was produced in bacteria and immobilized on Glutathione beads. The beads solution was then incubated with S2 cell lysates that expressed V5-tagged Yki or Yki (S97A) mutant
(both constructs were generated by X. Wei). Figure 3-1 showed that wild-type Yki but not Yki (S97A) mutant protein was pulled down by GST-Sd. GST protein was used as negative control and did not show any interaction with either Yki or Yki (S97A).

<table>
<thead>
<tr>
<th></th>
<th>Yki-V5</th>
<th>WT</th>
<th>S97A</th>
<th>WT</th>
<th>S97A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-Sd</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-1: Yki but not Yki (S97A) interacts with Sd. Bacterially produced GST (lane 1 and 2) or GST-Sd (lane 3 and 4) proteins were used in the in vitro pull-down assay. Yki-V5 (lane 1 and 3) or Yki (S97A)-V5 (lane 2 and 4) proteins were produced in transfected S2 cells. Yki proteins were detected with anti-V5 antibody and GST proteins were detected by Coomassie Blue staining.

3.3 Generation of UAS-Yki (S97A) and UAS-YAP (S94A/S127A) Transgenic Flies

To analyze the physiological functions of target genes in vivo, transgenic flies carrying UAS-Yki (S97A) (Sd-binding defective form) or UAS-YAP (S94A/S127A) (TEAD-binding defective form) (constructs made by X. Wei) were generated following the procedure mentioned in chapter 2. Ser 127 of YAP is the critical phosphorylation site
by Wts kinase and confers its inhibitory regulation of YAP activity (Zhao et al., 2007). YAP (S127A) functions as an active form.

6 lines of UAS-Yki (S97A) and 15 lines of UAS-YAP (S94A/S127A) were obtained. These transgenic flies allow the induction of transgene under the control of Gal4-UAS expression system. The UAS-Yki, UAS-YAP, UAS-YAP (S94A) and UAS-YAP (S127A) lines used in this study were previously generated by X. Wei.

3.4 Sd/TEAD-Binding Is Required for Yki/YAP to Promote Drosophila Eye Overgrowth

Overexpression of UAS-Yki, UAS-Yki (S97A), UAS-YAP, UAS-YAP (S94A), UAS-YAP (S127A) or UAS-YAP (S94A/S127A) in developing fly eyes was induced by GMR-Gal4 driver. It was reported before that ectopic Yki or YAP (S127A) expression produced enlarged and rough eye phenotype (Huang et al., 2005; Zhao et al., 2007) and (Figure 3-2 B, F compared to A). Overexpression of wild-type YAP also induced rough and large eye phenotype, which is much milder than YAP (S127A) as expected (Figure 3-2 D compared to F). Mutation of the critical Sd/TEAD binding site significantly compromised the growth-promoting activity of Yki/YAP (Figure 3-2 C, E, G compared to B, D, F, respectively).
Figure 3-2: Sd/TEAD-binding defective Yki/YAP is less potent in promoting *Drosophila* eye growth. SEM (scanning electron microscopy) images of adult fly eyes are presented. Genotypes of the fly tissues are:
(A) Wild-type (Canton S)
(B) GMR-Gal4/UAS-Yki-V5
(C) GMR-Gal4/UAS-Yki (S97A)-V5
(D) GMR-Gal4/UAS-Flag-YAP
(E) GMR-Gal4/UAS-Flag-YAP (S94A)
(F) GMR-Gal4/UAS-Flag-YAP (S127A)
(G) GMR-Gal4/UAS-Flag-YAP (S94A/S127A)

3.5 Sd/TEAD-Binding Is Required for Yki/YAP to Promote Drosophila Wing Overgrowth

Overexpression of UAS-Yki, UAS-Yki (S97A), UAS-YAP, UAS-YAP (S94A), UAS-YAP (S127A) or UAS-YAP (S94A/S127A) in developing fly wings was driven by C5-Gal4 (Figure 3-3 (1)). C5-Gal4 heterozygous flies were used as control. The sizes of wings from each genotype (n>20 for each genotype) were measured and normalized to the average wing size of control flies (Figure 3-3 (2)).

Eighty percent of the flies expressing Yki showed severely malformed wings and their wing area could not be measured (Figure 3-3 (1) B). The remaining twenty percent increased the wing size by 27% compared to the control (Figure 3-3 (1) C and (2). t-Test, P<0.05). Sd-binding defective mutation reduced the wing size enlargement to 19% and all wings were morphologically normal (Figure 3-3 (1) D and (2). t-Test, P<0.05).

Overexpression of human YAP in the flies enlarged the wing size by 14% (t-Test, P<0.05) and induced the developmental defects of longitudinal vein 4, which broke into three segments (Figure 3-3 (1) E and (2)). S94A mutation completely reversed the wing phenotypes associated with ectopic YAP expression (Figure 3-3 (1) F and (2)).

Consistent with the enhanced activity of YAP (S127A) compared to wild type YAP, YAP (S127A)-expressing wings were severely malformed with large air bubble formed between apical and basal layers and the wing size was immeasurable (Figure 3-3 (1) G). Single amino acid replacement of Ser 94 by Ala, however, completely reversed
this effect and produced wings with similar size and morphology as control flies (Figure 3-3 (1) H and (2)).

Put together, the wing tissue growth-promoting activity of Yki/YAP requires the binding of Sd/TEAD. The residual activity of Yki (S97A) indicates that Sd is not the only functional partner of Yki.
Figure 3-3: Sd/TEAD-binding defective Yki/YAP is compromised in promoting *Drosophila* wing growth. (1) SEM images of adult fly wings are presented. Arrows in (E) point to two gaps along the fourth longitudinal vein. Genotypes of the fly tissues are:

(A) Wild-type (C5-Gal4)

(B, C) C5-Gal4/UAS-yki-V5

(D) C5-Gal4/UAS- Yki (S97A)-V5

(E) C5-Gal4/UAS-Flag-YAP

(F) C5-Gal4/UAS-Flag-YAP (S94A)

(G) C5-Gal4/UAS-Flag-YAP (S127A)

(H) C5-Gal4/UAS-Flag-YAP (S94A/S127A)

(2) Relative wing size of each genotype listed above compared to control. The difference between the star-labeled groups and control group is statistically significant by t-Test, P<0.05. C5>Yki(S97A) wings are significantly smaller than C5>Yki wings (t-Test, P<0.05).
3.6 Sd/TEAD-Binding Is Essential for Yki/YAP to Induce Extra Interommatidial Cells

Based on the adult eye and wing phenotypes shown above, it is clear that Yki/YAP possess the tissue growth promoting activity and this activity is dependent on Sd/TEAD binding. A signature activity of Hippo signaling is to regulate the cell number by coordinately modulating cell proliferation and cell death. Next, we wanted to examine whether Sd/TEAD binding is required for Yki/YAP to induce extra cell numbers.

Wild-type flies have fixed number of interommatidial cells in mid-pupal eye discs due to the elimination of extra cells by apoptosis (Figure 3-4, A). Previous studies have shown that loss of Hippo signaling results in the accumulation of extra interommatidial cells in mid-pupal eye discs due to the defective apoptosis (Udan et al., 2003; Willecke et al., 2006). This provides a useful model to study the importance of Sd/TEAD binding in Yki/YAP’s effect on cell numbers. UAS-Yki, UAS-Yki (S97A), UAS-YAP, UAS-YAP (S94A), UAS-YAP (S127A) or UAS-YAP (S94A/S127A) were expressed in developing eyes by GMR-Gal4 and the mid-pupal eye discs were stained with anti-Discs large (Dlg) antibody to outline the cells (Figure 3-4). Overexpression of Yki, YAP or YAP (S127A) all induced numerous amounts of interommatidial cells, where Yki and YAP (S127A) were much more potent than wild type YAP (Figure 3-4 B, D, F. Images collected by X. Wei). Mutation of the critical Sd/TEAD binding site dramatically reduced their potential in increasing cell numbers (Figure 3-4 C, E, G compared to B, D, F, respectively).
Figure 3-4: Sd/TEAD-binding defective Yki/YAP is compromised in inducing extra interommatidial cells. Mid-pupal eye discs were stained with Discs large (Dlg) antibody to outline cells. Arrows point to interommatidial cells. Genotypes of the fly tissues are:

(A) Wild-type (Canton S)
(B) GMR-Gal4/UAS-Yki-V5
(C) GMR-Gal4/UAS- Yki (S97A)-V5
(D) GMR-Gal4/UAS-Flag-YAP
(E) GMR-Gal4/UAS-Flag-YAP (S94A)
(F) GMR-Gal4/UAS-Flag-YAP (S127A)
3.7 Sd/TEAD-Binding Is Critical for Yki/YAP to Induce Clone Expansion

Imaginal discs are clusters of epithelial cells that invaginate from the embryo ectoderm and increase their size by cell division during larval development (Cohen et al., 1993). Clones of disc cells homozygous for gain of function of specific gene can be generated via mitotic recombination using FLPout technique (Figure 3-5). In the FLPout system, the ubiquitous Actin promoter is joined to the Gal4 coding sequence by an interruption cassette, which contains a marker gene “w+” and the transcriptional termination signal flanking by two FLPase recombination target (FRT) sites. Heat-shock-induced expression of Flippase triggers the recombination between these two FRT sites and releases the cassette, leading to the expression of Gal4. Gal4 protein in turn drives the expression of UAS-coupled genes, including the UAS-GFP marker gene and UAS-transgene. The founder cells continue to divide and give rise to clusters of cells of the same genotype, called clones. Since not all cells respond to heat shock, the cells that do not undergo mitotic recombination are free of the expression of target gene as well as GFP marker gene. As a result, clones of GFP-positive cells expressing target gene are formed in fluorescence free background.
Figure 3-5: Scheme of FLPout technique. FLP-FRT system and UAS-Gal4 system are combined in FLPout technique. Heat-shock-induced expression of yeast FLP recombinase triggers the recombination of two FRT sites on the same chromosome and releases the interruption cassette in-between. This leads to the expression of Gal4 protein and subsequently activates the UAS-gene expression, such as GFP marker or other transgenes of interest.

To induce overexpression of Yki/YAP mutant genes in clones of cells, males carrying UAS-Yki, UAS-Yki (S97A), UAS-YAP (S127A) or UAS-YAP (S94A/S127A) transgenes were crossed with females of w; hsFLP; act> w+ > Gal4; UAS-GFP/TM6B. For the control experiment, 176 males were used. Late third instar wing discs were examined and all clones were positively labeled with GFP (Figure 3-6). Both Yki and YAP-S127A were potent in promoting cell division and therefore produced larger clones than control (Figure 3-6 B, D compared to A). Moreover, the whole discs were generally
larger than wild-type discs (Figure 3-6 B, D compared to A). However, neither Yki (S97A) nor YAP (S94A/S127A) showed a similar level of growth-promoting activity (Figure 3-6 C, E).

Figure 3-6: Sd/TEAD-binding defective Yki/YAP is compromised in clone expansion. Clones of cells overexpressing target genes were generated by FLPout technique and positively marked with GFP. Late third instar wing discs were shown. The genotypes of the fly tissues are:
(A) hsFLP/+; act>w>Gal4, UAS-GFP/+  
(B) hsFLP/+; act>w>Gal4, UAS-GFP/UAS-Yki-V5  
(C) hsFLP/+; act> w>Gal4, UAS-GFP/UAS-Yki (S97A)-V5  
(D) hsFLP/+; act> w>Gal4, UAS-GFP/UAS-Flag-YAP (S127A)  
(E) hsFLP/+; act> w>Gal4, UAS-GFP/UAS-Flag-YAP (S94A/S127A)
3.8 \textit{s}d and \textit{y}ki Functionally Interact to Promote Tissue Growth

As we showed before, Sd and Yki physically interact and Sd/TEAD binding is critical for Yki/YAP to promote tissue growth. Next, we wanted to examine whether Sd and Yki function in the same signaling pathway \textit{in vivo}. To do this test, different combinations of gain or loss of function of \textit{sd} or \textit{yki} were generated and the adult eye phenotypes were analyzed.

3.8.1 A Strong Loss-of-Function Allele of \textit{s}d Suppresses Gain-of-Yki Phenotype

Overexpression of Yki driven by GMR-Gal4 had been shown to produce enlarged and rough eyes (Zhao et al., 2007) and (Figure 3-7 B). If \textit{sd} does functionally interact with \textit{yki}, the change in \textit{sd} gene dosage might affect the phenotype of gain of \textit{yki} function. First of all, we examined the effect of reduction of \textit{sd} dosage. \textit{sd}^{l2} allele carries a 157 bp deletion and the homozygous \textit{sd}^{l2} is larval lethal (Srivastava et al., 2004). Female flies heterozygous for \textit{sd}^{l2} did not show any abnormality in adult eyes (Figure 3-7 D). When introduced into the gain of \textit{yki} genetic background (a strong UAS-Yki transgenic line was used in this test), \textit{sd}^{l2} obviously suppressed the large and rough eye phenotypes (Figure 3-7 C compared to B).
Figure 3-7: A strong sd mutant allele suppresses the large and rough eye phenotype associated with Yki overexpression. (A) Wild type adult fly eyes. (B) Overexpression of Yki by GMR-Gal4 produces enlarged and rough eyes. (D) Flies hemizygous for a strong sd mutant allele show normal eyes. (C) This strong loss of sd allele suppresses the adult eye overgrowth phenotype of Yki overexpression.

3.8.2 Sd Overexpression Enhances Gain-of-Yki Phenotype

Next, we examined the effect of increase of sd gene dosage in Yki overexpression phenotype. UAS-Sd and UAS-Yki were coexpressed by GMR-Gal4 and the adult eye phenotype was observed. A weak UAS-Yki transgenic line was used, which produced slightly bigger eyes with rough surface when overexpressed by GMR-Gal4 (Figure 3-8 B). Overexpression of Sd by GMR-Gal4 caused smaller eye size, presumably due to a dominant-negative effect (Simmonds et al., 1998) and (Figure 3-8 D). This is consistent with the phenomena of TEAD overexpression in mammalian cells. Overexpression of TEAD1 in cells that normally express TEAD proteins result in transcription repression, indicating the titrating out of a coactivator activity (Xiao et al., 1991). This reduced eye phenotype was suppressed when Yki was coexpressed with Sd (Figure 3-8 C compared to D). This is supportive of the role of Yki as the functional partner of Sd because ectopic
Yki was able to suppress the dominant-negative effect of Sd very likely due to the release of out-titrated ratio between Sd and its coactivating factor, Yki. On the other hand, coexpression of Sd and Yki enhanced the eye phenotype of Yki overexpression alone, supporting the collaborative growth-promoting activity of these two proteins (Figure 3-8 C compared to B). When a strong UAS-Yki line was used, Sd coexpression led to 100% late pupal lethality and the pupae eyes overgrew vigorously (Figure 3-8 B’ and C’).

Figure 3-8: Coexpression of Sd and Yki enhances eye phenotype of Yki overexpression. (A) Wild type adult fly eyes. (B) Overexpression of a weak UAS-Yki transgene by GMR-Gal4 produces slightly enlarged and rough eyes. (D) Ectopic expression of Sd results in smaller eyes due to dominant-negative effect. (C) Gain of function of both Sd and Yki enhances the eye overgrowth induced by Yki overexpression, and suppresses the dominant-negative effect of Sd overexpression. Similar effect was seen when a strong
UAS-Yki line was used. (B’) Overexpression of Yki shows dramatic overgrowth of eye tissue. (C’) Coexpression of Sd with the strong Yki transgene results in 100% late pupal lethality and the dead pupae shows marvelous overgrowth of eye tissue.

3.8.3 yki Mutation Enhances the Dominant-Negative Effect of Sd Overexpression

As mentioned before, the ectopic sd expression causes smaller rough eye phenotype as the result of unbalanced molecule ratio between Sd and its co-factor(s). Under this genetic background, a decrease of the co-factor(s)’s gene dosage is expected to further askew this out-titration and result in the enhancement of the dominant-negative effect. To test whether Yki is the co-factor of Sd, loss of one yki allele was introduced in Sd overexpressing flies (Figure 3-9). Flies heterozygous of yki gene were normal (data not shown). When combined with Sd overexpression, however, all flies died at late pupal stage and had no eyes (Figure 3-9 C). This was a strong enhancement of growth defects compared to Sd overexpression alone (Figure 3-9 C compared to B).

Figure 3-9: Reduction of yki level enhances the dominant-negative effect of Sd overexpression. (A) Wild type adult fly eyes. (B) Ectopic expression of Sd results in smaller rough eyes. (C) Loss of one yki allele enhances Sd overexpression phenotype and
causes late pupal lethality. Those dead pupae didn’t have eye tissue in the area where the retina is normally expected to grow (as denoted by arrow).

3.8.4 yki Mutation Dominantly Enhances the Hypomorphic Wing Phenotype of sd

sd is an essential gene and the flies lacking sd function could not survive. To better understand the developmental function of sd gene and its relationship with yki, a hypomorphic allele of sd (sd1) was used (Campbell et al., 1991). Homozygous females (data not shown) or hemizygous males of sd1 caused notches in the adult wing margins, called “scalloped” wings (Figure 3-10 B). The yki null mutant is recessive and homozygous yki−/− is lethal at early stage of embryonic development (Huang et al., 2005). The flies heterozygous for yki +/- did not show any growth defects (data not shown). When one allele of yki was removed from sd1 hemizygous male flies, the scalloped wing phenotype was dramatically enhanced (Figure 3-10). The notched wing phenotypes of hemizygous sd1 males exhibited some variations in the severity (Figure 3-10 (1)), with more than 90 percent of wings showing the mildest 1° defect while only 7 percent showing 2° defect and 2 percent showing the severest 3° defect (Figure 3-10 (2)). Introduction of one null yki allele distinctly shifted the distribution curve to the right, with about 30 percent of wings displaying the most severely defective morphology and only 60 percent of wings staying in the mildest 1° range (Figure 3-10 (2)). It implies that the function of sd1 is sensitive to the in vivo level of yki.
Figure 3-10: Reduction of \( yki \) enhances the notched wing phenotype of \( sd \) hypomorphic allele. (1) The defects in wings range from mild cuts at the margin to strongly notched and shrunk tiny wings. The severity of phenotype was classified using an arbitrary scale of 1° to 3° (B-D). 0° represents wild-type wings (A). (2) Loss of one allele of \( yki \) (n=169) enhances the defective wing phenotype associated with hemizygous \( sd \) hypomorphic allele (n=298).
3.9 Chapter Summary

In this chapter, we identified Sd as the interacting partner of Yki to promote tissue growth. *In vitro* pull-down assay supported the physical interaction between Sd and Yki. Moreover, the work of mammalian system from Guan’s lab demonstrated that Ser 94 of YAP is the critical TEAD-binding site (Zhao et al., 2008). Consistent with this, the interaction between Sd and Yki was abolished when the corresponding Ser 97 of Yki was mutated into Ala.

Next, the functional significance of Sd/TEAD binding was examined by performing the phenotypic analysis of several Yki/YAP mutants overexpressing flies. The adult phenotypes showed that Sd/TEAD binding defective Yki/YAP is less potent in promoting eye and wing tissue growth. The mid-pupal eye discs of wild type flies have fixed number of interommatidial cells due to the elimination of extra cells by apoptosis. Flies overexpressing Yki or YAP possess numerous extra interommatidial cells by inhibiting cell death. Loss of Sd/TEAD binding, however, dramatically reduces their activity in inducing extra number of cells. In the end, the activity of Yki/YAP mutants in clonal expansion was tested. FLP/FRT recombination system was combined with UAS-Gal4 expression system to induce target gene expression in clones of cells. The growth-promoting transgenes will produce larger and more clones whereas the growth-inhibiting transgenes will generate smaller and less clones. As expected, Sd/TEAD binding is essential for Yki/YAP to promote clone expansion.

After proving the importance of Sd/TEAD in Yki/YAP-dependent biological functions, the genetic interaction between *sd* and *yki* was established with strong evidence. Overexpression of Yki was reported to produce enlarged and rough eye phenotype (Zhao et al., 2007). This eye overgrowth phenotype was suppressed by a strong loss of function allele of *sd* and significantly enhanced by Sd coexpression. On the other hand, ectopic Sd expression caused smaller and rough eyes due to dominant-negative effect (Simmonds et al., 1998). This dominant-negative effect is thought to be resulted from the out-titrated ratio between Sd and its interacting factor(s). Loss of one *yki* allele dramatically enhanced the Sd-overexpression-related growth defects, which is
supportive of the identity of Yki as the functional partner of Sd. Moreover, the observation that loss of one yki allele enhanced the hypomorphic phenotypes of sd mutant further implies their relationship as functional partners.

Although Sd is essential for Yki to promote eye and wing tissue growth, the overexpression of Sd-binding defective Yki still shows obvious overgrowth compared to wild-type flies. Moreover, different from the ubiquitous requirement of Yki for tissue growth, Sd is not needed in all imaginal tissues (Goulev et al., 2008). It indicates that additional transcription factor(s) might be utilized by Yki to control growth.
Chapter 4
Functional Analysis of WW Domains of Yki/YAP

4.1 Introduction

Hippo signaling pathway negatively regulates Yki activity by phosphorylating and retaining Yki in the cytoplasm, therefore blocking the Yki-mediated target gene transcription (Dong et al., 2007). YAP, the mammalian homolog of Yki, is regulated in the similar way, where its phosphorylation by mammalian Lats kinase promotes 14-3-3 binding and results in cytoplasmic retention and functional inhibition of YAP (Zhao et al., 2007). YAP was implicated as an oncogene since the discovery of the amplicon of murine locus 9qA1 in mouse mammary tumors, where YAP is the only known gene (Overholtzer et al., 2006). Moreover, its syntenic locus in human genome, 11q22, was also found to be amplified in various human cancers (Hermsen et al., 2005; Lambros et al., 2005; Snijders et al., 2005). In spite of the well-defined growth-promoting activity of Yki/YAP, little is known about the molecular mechanisms underlying their regulation.

Yki is a transcription coactivator lacking DNA binding domain, which needs transcription factor (s) to mediate its target gene regulation. Recent studies have shown that Sd interacts with Yki and is required for Yki-dependent tissue growth (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b). The mammalian homologue, TEAD, was also shown to mediate YAP-dependent gene induction and growth control (Zhao et al., 2008). However, Yki and Sd are not identically required for fly development. Whereas sd is specifically required for the wing and neuronal development (Campbell et al., 1992), yki seems to be needed for all imaginal discs (Huang et al., 2005). Within wing imaginal discs, yki clones die in the entire wing disc whereas sd is only essential in the wing pouch area (Goulev et al., 2008). Moreover, overexpression of Sd-binding defective Yki (S97A) still induces overgrowth of fly eye and wing tissues (Zhao et al., 2008). Expression of YAP-dependent target genes is still stimulated by TEAD-binding defective YAP (S94A)
Therefore, additional transcription factor(s) other than Sd must be used by Yki/YAP to exert their growth control activity.

The WW domain, also known as the WWP or rsp5 domain, comprises of around 40 amino acids with two signature tryptophan (W) residues spaced 20 to 22 amino acids apart (Sudol and Hunter, 2000). WW domain functions as an interaction module binding specifically to the proline-rich sequences exemplified by PPxY (PY) motifs (Andre and Springael, 1994; Chen and Sudol, 1995). Many of the PY motif-containing proteins are transcription factors and this PY motif has been indicated to mediate transcription activation by interacting with WW domain-containing proteins (Yagi et al., 1999). Yki/YAP contains either one or two WW domains depending on different splicing patterns (Huang et al., 2005; Sudol et al., 1995). Several transcription factors, such as ErbB4, RUNX and P73, have been shown to bind to YAP via PY-WW domain interaction (Basu et al., 2003; Strano et al., 2001). However, it is not known whether this interaction mediates YAP-dependent gene induction and growth control. On the other hand, WW domains of Yki have been shown to be required for the Yki-Wts interaction, indicating the putative inhibitory effect conferred by WW domains (Huang et al., 2005).

Zhao et al. found that WW domain of YAP is dispensable for its inhibition by Lats kinase. However, it is essential for YAP-induced cell proliferation and a subset of target gene expression (Zhao et al., 2009). In vivo functional analysis of WW domain was performed in Drosophila and showed that WW domain is critical for Yki/YAP to promote tissue growth, to increase cell number and to stimulate clone expansion. The two WW domains of Yki synergistically act to mediate its growth-promoting activity.

**4.2 Generation of UAS-Yki (W1W2), UAS-Yki (WW1), UAS-Yki (WW2) and UAS-YAP (S127A/W1W2) Transgenic Flies**

To study the biological functions of Yki/YAP mutant genes in vivo, transgenic flies carrying UAS-Yki (W1W2) (mutating both WW domains of Yki), UAS-Yki (WW1) (mutating WW1 domain of Yki), UAS-Yki (WW2) (mutating WW2 domain of Yki), or UAS-YAP (S127A/W1W2) (double mutant for Ser127 and WW domains) were
generated (DNA constructs made by X. Wei). Multiple lines were generated for each genotype. UAS-YAP (W1W2) line used in this chapter was previously generated by X. Wei. Yki mutant constructs with critical sites of WW domain(s) replaced by Alanine were generated by X. Wei. Yki (WW1) replaces Tryptophan 292 and Proline 295 with Alanine (W292A and P295A). Yki (WW2) replaces Trp 361 and Pro 364 with Ala (W361A and P364A). Yki (W1W2) combines the mutations of both WW domains. YAP mutants used were gifts from Guan’s lab.

4.3 WW Domains Are Important for Yki/YAP to Promote Drosophila Eye Overgrowth

As known before, overexpression of Yki or YAP (S127A) by GMR-Gal4 produces enlarged and rough eye. Mutation of WW domains in Yki or YAP (S127A) dramatically suppressed the tissue overgrowth phenotype (Figure 4-1 C, E compared to B, D). However, the fly eyes overexpressing Yki (W1W2) or YAP (S127A/W1W2) still showed rough surface with slightly bigger size (Figure 4-1 C, E compared to A), which suggested that WW domains are critical for the growth-promoting activity of Yki/YAP but are not the only function-conferring domains.
Figure 4-1: WW domains are important for Yki/YAP to induce *Drosophila* eye overgrowth. Genotypes of the fly tissues are:

(A) Wild-type (Canton S)
(B) GMR-Gal4/UAS-Yki-V5
(C) GMR-Gal4/UAS- Yki (W1W2)-V5
(D) GMR-Gal4/UAS-Flag-YAP (S127A)
(E) GMR-Gal4/UAS-Flag-YAP (S127A/W1W2)
4.4 WW Domains Are Critical for Yki/YAP to Promote Drosophila Wing Overgrowth

Overexpression of Yki, YAP or YAP (S127A) by C5-Gal4 has been shown to produce fly wings with larger size and/or abnormal morphology (Figure 3-3, 4-2). Mutation of WW domains in Yki significantly reduced the wing enlargement from 27% to 7% (Figure 4-2 (1) D and (2). t-Test, P<0.05). This suppression is stronger than the S97A mutation of Yki mentioned in previous chapter, which reduced the wing size increase from 27% to 19% (Figure 3-3). This implies that WW domains of Yki might mediate a stronger function independent of Sd-binding to promote tissue growth.

Mutations of WW domains in YAP/YAP (S127A), however, completely reversed the wing overgrowth phenotype and morphology defects, similar to S94A mutation (Figure 4-2 (1) F, H and (2); Figure 3-3). The lack of residual growth-promoting effect of YAP (W1W2) or YAP (S127A/W1W2) might be wing tissue-specific since the YAP (S127A/W1W2)-expressing eyes are rough and enlarged (Figure 4-1 E). The other possibility is that the additional mammalian factor(s) required for YAP mutants to promote tissue growth is absent in Drosophila.
Relative Wing Size

(1).

A  WT

B  Yki

C  Yki

D  Yki (W1W2)

E  YAP

F  YAP (W1W2)

G  YAP (S127A)

H  YAP (S127A/W1W2)

(2).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative Wing Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5-GAL4</td>
<td>27%</td>
</tr>
<tr>
<td>C5-Yki</td>
<td>7%</td>
</tr>
<tr>
<td>C5-YAP (W1W2)</td>
<td>14%</td>
</tr>
<tr>
<td>C5-YAP (S127A)</td>
<td>-2%</td>
</tr>
<tr>
<td>C5-YAP (S127A/W1W2)</td>
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</tbody>
</table>

* indicates significant difference from control.
Figure 4-2: WW domains are important for Yki/YAP to promote *Drosophila* wing overgrowth. (1) SEM images of adult fly wings are presented. Genotypes of the fly tissues are:

(A) Wild-type (C5-Gal4)
(B, C) C5-Gal4/UAS-yki-V5
(D) C5-Gal4/UAS- Yki (W1W2)-V5
(E) C5-Gal4/UAS-Flag-YAP
(F) C5-Gal4/UAS-Flag-YAP (W1W2)
(G) C5-Gal4/UAS-Flag-YAP (S127A)
(H) C5-Gal4/UAS-Flag-YAP (S127A/W1W2)

(2) Relative wing size of each genotype listed above compared to control. The difference between the star-labeled groups and control group is statistically significant by t-Test, P<0.05. C5>Yki(WW1WW2) wings are significantly smaller than C5>Yki wings (t-Test, P<0.05).

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4.5 WW Domains Are Important for Yki/YAP to Induce Extra Interommatidial Cells

As mentioned in previous chapter, interommatidial cells of mid-pupal eye discs provide a good model to study the regulation of cell number. Moreover, the modulation of interommatidial cell number has been widely used as readout of Hippo signaling. Therefore, the examination of interommatidial cell number was used to analyze the functional significance of WW domains of Yki/YAP. Overexpression of various Yki/YAP mutants was driven by GMR-Gal4 and Dlg staining was performed to outline cells in mid-pupal stage (Figure 4-3). Induction of extra interommatidial cells by overexpressing Yki, YAP or YAP (S127A) was shown before in Figure 3-4 and again here in Figure 4-3. Mutation of WW domains dramatically decreased the Yki/YAP potential in inducing extra cell number, which was less prominent in YAP (W1W2) mutant (Figure 4-3 C, E, G compared to B, D, F, respectively).
Figure 4-3: WW domains-mutated Yki/YAP is less potent in inducing extra interommatidial cells. Cells were outlined by Dlg staining. Arrows point to interommatidial cells. Genotypes of the fly tissues are:

(A) Wild-type (Canton S)
(B) GMR-Gal4/UAS-Yki-V5
(C) GMR-Gal4/UAS- Yki (W1W2)-V5
(D) GMR-Gal4/UAS-Flag-YAP
(E) GMR-Gal4/UAS-Flag-YAP (W1W2)
(F) GMR-Gal4/UAS-Flag-YAP (S127A)
4.6 WW Domains Are Important for Yki/YAP to Promote Clone Expansion

The functional significance of WW domains was further investigated by clonal analysis. Clones of cells overexpressing Yki/YAP and their derivatives were generated by FLPout technique and their ability to promote clone expansion in wing discs was monitored. Compared to wild type controls, ectopic Yki or YAP (S127A) strongly stimulated clone expansion so that individual clones and whole wing discs were larger (Figure 4-4 B, D compared to A). Mutations of WW domains, however, greatly suppressed the Yki and YAP (S127A) activity as both the clone size and wing disc size were similar to those of controls (Figure 4-4 C, E compared to A).
Figure 4-4: WW domains are important for Yki/YAP to promote clonal expansion. Clones of cells overexpressing target genes were generated by FLPout technique and positively marked with GFP. Late third instar wing discs were shown. The genotypes of the fly tissues are:

(A) hsFLP/+; act>"Gal4, UAS-GFP/+  
(B) hsFLP/+; act>"Gal4, UAS-GFP/UAS-Yki-V5  
(C) hsFLP/+; act>"Gal4, UAS-GFP/UAS-Yki (W1W2)-V5  
(D) hsFLP/+; act>"Gal4, UAS-GFP/UAS-Flag-YAP (S127A)  
(E) hsFLP/+; act>"Gal4, UAS-GFP/UAS-Flag-YAP (S127A/W1W2)
4.7 Two WW Domains of Yki Additively Act to Mediate Its Activity

Overexpression of Yki mutants for single WW domain was driven by GMR-Gal4 and the eye phenotypes were observed (Figure 4-5). Ectopic Yki (WW1) produced slightly enlarged eyes with multiple bristles formed in most of sockets (Figure 4-5 B. The strongest I° severity). The eye phenotypes associated with ectopic Yki (WW2) were weaker compared to Yki (WW1), with multiple bristles only formed in some of the sockets (Figure 4-5 C. II° severity). When both WW domains were mutated, however, formation of multiple bristles was rarely seen and the eye size was closer to wild type controls (Figure 4-5 D. The mildest III° severity). Therefore, the two WW domains of Yki have additive effect in ectopic Yki-induced eye phenotypes.

Figure 4-5: Two WW domains of Yki have additive effect. Formation of multiple bristles in single socket is most frequently seen in Yki (WW1) expressing eyes (B) followed by Yki (WW2) (C), and is hardly seen in Yki (W1W2) expressing eyes (D). The severity of eye phenotype is classified from I (the strongest) to III (the weakest) degrees. Genotypes of the fly tissues are:
(A) Wild-type (Canton S); (B) GMR-Gal4/UAS-Yki (WW1)-V5; (C) GMR-Gal4/UAS-Yki (WW2)-V5; (D) GMR-Gal4/UAS-Yki (W1W2)-V5.
4.8 Chapter Summary

In this chapter, \textit{in vivo} function of WW domains of Yki/YAP was examined in \textit{Drosophila} larvae, pupae as well as adults. Mutation of both WW domains dramatically reduced the potential of Yki/YAP to promote eye or wing tissue overgrowth. These adult phenotypes are supportive of the positive role of WW domains in Yki/YAP-mediated growth control. To dissect the function of WW domains in specific biological processes, more sensitive assay was performed in earlier developmental stages, such as third instar larvae and mid-pupae. WW domain was shown to be critical for Yki/YAP to induce extra interommatidial cells in mid-pupal eye discs and also important for Yki/YAP to stimulate clone expansion in late third instar larvae wing discs. Based on the fact that double WW domain mutants have weaker eye phenotypes than either WW1 or WW2 domain mutant, the two WW domains of Yki might have additive effect in promoting eye tissue growth.

In summary, WW domain is positively required for Yki/YAP activity in an Sd/TEAD-independent way (Oh et al., 2009; Zhao et al., 2009). The future direction will be the identification of the additional transcription factor(s) that acts through WW domain to mediate Yki/YAP function.
Chapter 5

Crosstalk between Hippo and Akt Signaling Pathways

5.1 Introduction

In the developing *Drosophila* wing disc, cell growth can be uncoupled from cell division (Neufeld et al., 1998). When cell proliferation rate is accelerated by overexpressing specific cell-cycle regulators, such as dE2F, in cell clones or wing compartments, cell growth is unaffected and the cells divide at a smaller size. Therefore, although the cell numbers were increased 4- to 5- folds, clone or compartment sizes were not changed much (Neufeld et al., 1998). In other words, cell division acceleration is insufficient to drive growth (Johnston et al., 1977; Nurse, 1975). Loss of Hippo signaling, however, has been shown to cause dramatic overgrowth in clones or tissues. It has been well known that mutations of Hippo signaling components stimulate cell proliferation and inhibit apoptosis, thereby increasing cell numbers. It remains unclear how the increase in cell mass is coupled to cell cycle acceleration to achieve the clonal or tissue overgrowth in Hippo mutant animals. Is there any other signaling pathway(s) or is Hippo pathway per se involved in maintaining the appropriate cell growth rate? How does Hippo signaling perform the function of cell growth control? If other signaling pathway(s) participates in this process, what is their identity and how does Hippo signaling communicate with them? These are the questions I am going to address in this chapter.

Protein synthesis comprises the main part of animal growth during development. Among the signaling pathways which regulate the protein translation apparatus, insulin receptor (Inr)/Akt signaling pathway (the term “Akt signaling” is used in the writing below) is the most important one. Akt signaling pathway is known to play important role in growth control by regulating cell proliferation, cell growth and apoptosis and also in other biological processes, such as metabolism, reproduction and longevity (Oldham and Hafen, 2003). The schematic outline of *Drosophila* Akt signaling pathway is shown in
Figure 5-1. In response to ligand binding, insulin receptor (Insr) is activated to recruit Chico, *Drosophila* homolog of Insulin Receptor Substrate (IRS), and the lipid kinase PI3K (phosphoinositide 3-kinase) to the plasma membrane (Bohni et al., 1999; Leevers et al., 1996; Weinkove et al., 1999). PI3K functions by converting PIP2 (phosphatidylinositol (4,5)-bisphosphate) into PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) via phosphorylation. This effect is reversed by lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10), which dephosphorylates PIP3 back to PIP2 (Maehama and Dixon, 1999). PIP3 at the membrane recruits pleckstrin homology (PH) domain-containing protein serine/threonine kinases, Akt (also called protein kinase B, PKB) and PDK1 (phosphoinositide-dependent kinase), to colocalize (Brazil and Hemmings, 2001; Coffer et al., 1998). Upon Ser/Thr phosphorylation by PDK1 and TOR (target of rapamycin), Akt kinase is activated (Alessi et al., 1997; Sarbassov et al., 2005). Activated Akt inhibits Tsc1-2 complex (tuberous sclerosis complex 1 and 2), which in turn activates TOR complex 1 (TORC1) by relieving its indirect inhibition by Tsc1-2 complex (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). TOR also exists in another rapamycin-insensitive complex, TOR complex 2 (TORC2), which acts upstream of Akt as a positive regulator (Loewith et al., 2002). Activated TORC1 subsequently phosphorylates 4E-BP (4E-binding protein), which blocks its function as inhibitor of cap-dependent translation (Lawrence and Abraham, 1997). TOR and PDK1 also activate ribosomal S6 kinase (S6K), thereby controlling the activity of protein translation machinery (Dufner and Thomas, 1999). Mutations of the positive components or overexpression of the inhibitory components of Akt pathway result in reduced cellular growth rate (Hietakangas and Cohen, 2009).
Figure 5-1: Akt signaling pathway in *Drosophila*. The branch of cell growth regulation by Akt signaling is shown.

To investigate whether there is interplay between Hippo signaling and Akt signaling pathways, we started with the examination of the effects of loss of Hippo in Akt expression as well as activity. To generate positively labeled mosaic clones mutant for Hippo pathway components, MARCM (Mosaic analysis with a repressible cell marker) system was used (Figure 5-2). Loss of Hippo signaling increased Akt mRNA and protein levels and stimulated Akt activity. Consistently, phosphorylation level of 4E-BP protein was also elevated in loss of Hippo context. Moreover, the induction of Akt protein level by loss of *mats* is dependent on *yki*. No obvious effect on Pten protein level was observed in Hippo signaling mutant clones. Adult wing phenotype indicated the genetic interaction between *akt* and *mats* genes. Genetic analysis showed that *mats* mutant-induced clonal
overgrowth can occur when the expression of \textit{akt} or \textit{pi3k} is greatly reduced, suggesting that Hippo signaling might target components downstream of Akt for regulating cellular growth.

Figure 5-2: Mosaic analysis with a repressible cell marker (MARCM). Conventional FRT/FLP system is combined with Gal4/UAS expression system and Gal4/Gal80 antagonizing system. Expression of Gal80 suppresses the Gal4-dependent UAS-marker gene induction (such as UAS-GFP). Heat shock-induced expression of FLP recombinase drives somatic recombination between two FRT sites present at homologous chromosomal locations, generating daughter cells homozygous for gene mutation while eliminating Gal80 repressor at the same time. Expression of GFP marker, therefore, is induced in homozygous mutant cells due to the absence of GAL80 protein. In the case of epistasis analysis, UAS-gene B can be introduced into the homozygous mutant background of gene A and their cooperative effects could be analyzed.
5.2 Akt Activity Is Negatively Regulated by Hippo Signaling

5.2.1 P-Akt Level Is Decreased in Regions Expressing Constitutively Active Mats

Mammalian Akt kinase is activated by phospholipid binding and additional phosphorylation at Thr308 by PDK1 and at Ser473 by Tor (Bayascas and Alessi, 2005). Phospho-specific antibody against Akt (Ser473) was obtained from Cell Signaling Technology (Cat. No. 4060) and was proven to be able to cross-react with Drosophila Akt when phosphorylated at Ser473. This antibody was therefore used to examine the activity of Akt in developing tissues of Drosophila conferring different kinds of mutations of Hpo signaling.

A constitutively active form of Mats was generated by Li-Lun Ho by tagging myristoylation signal peptide (Myr) at the N-terminus of Mats-GFP (Ho et al., 2010). Ectopic expression of Myr-Mats-GFP in the posterior regions of larva wing discs was driven by engrailed-Gal4 (en-Gal4). Third instar larva wing discs were utilized for immunostaining. Level of phospho-Akt (p-Akt) was downregulated in Myr-Mats-GFP overexpressing area compared to the wild type anterior region of the same wing disc (Figure 5-3 E-H). To prove that the suppression of Akt activity is Myr-Mats-GFP specific effect, overexpression of GFP transgene driven by en-GAL4 was used as control and no change of p-Akt staining was observed (Figure 5-3 A-D).
Figure 5.3: Akt activity is suppressed in regions overexpressing Myr-mats-GFP. (E) Myr-mats-GFP is expressed in posterior region (to the right) of larva wing disc by en-Gal4. (F-H) Staining of p-Akt is downregulated in Myr-mats-GFP overexpressing area. (A) GFP transgene is expressed by en-Gal4 as control. (B-D) No change of p-Akt staining is observed in GFP overexpressing region.

5.2.2 P-Akt Level Is Upregulated in Loss of mats, Loss of wts or Loss of hpo Clones

As shown above, Akt activity was suppressed in regions of developing wing tissues which express constitutively active form of Mats protein. To investigate how loss of Hippo signaling might affect Akt activity, p-Akt level was examined in clones of cells mutant for mats, wts or hpo. To generate loss of mats or wts clones, males of w;UAS-GFP; FRT82B mats^{194a}/S.T. or males of w; UAS-GFP; FRT82 wts^{-1}/S.T. were mated with females of y^{w}; hsFLP; tub-Gal4, FRT82B tub-Gal80/TM6B. For control crosses, males of w; FRT82B P[w^{+}] were mated with females of y^{w}; hsFLP, UAS-GFP; tub-
Gal4, FRT82B tub-Gal80/TM6B. P-Akt level was increased in both loss of mats and loss of wts clones but not in control clones as shown in Figure 5-4.

Figure 5-4: Akt activity is upregulated in loss of mats or loss of wts clones. (D) Loss of mats and (G) loss of wts clones were generated by MARCM and positively marked by GFP. (E, F, H, I) P-Akt staining is increased in both loss of mats and loss of wts clones. (A-C) 82B control clones do not show difference in the p-Akt level.
To generate loss of \textit{hpo} clones, males of \textit{w; FRT42D hpo}^{\text{BF33}}/\text{S.T.} were crossed with females of \textit{hsFLP122, UAS-GFP; FRT42D tub-Gal80; tub-Gal4/TM6B}. For control crosses, males of \textit{w; FRT42D P[w+] were crossed with females of hsFLP122, UAS-GFP; FRT42D tub-Gal80; tub-Gal4/TM6B}. Similarly to loss of \textit{mats} or loss of \textit{wts} clones, loss of \textit{hpo} function induced Akt activity, which was not seen in control clones (Figure 5-5).

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Figure 5-5: Akt activity is increased in loss of \textit{hpo} clones. (D) Loss of \textit{hpo} clones were generated by MARCM and positively marked by GFP. (E, F) Level of p-Akt is upregulated in loss of \textit{hpo} clones. (A-C) P-Akt staining is not changed in 42D control clones.
5.2.3 P-Akt Level Is Elevated in Yki Overexpressing Clones

Mats, Wts and Hpo are core components of kinase cascade of Hippo signaling pathway. Loss of function of any of these three genes has been shown to be able to enhance Akt activity (Figure 5-4, 5-5). To better understand the regulatory mechanism underneath, it would be interesting to see whether Yki, transcriptional coactivator acting downstream of Hippo signaling, is capable of regulating Akt activity. Yki overexpressing clones were generated by crossing males of w`; UAS-yki with females of w`, hsFLP; act>w`>Gal4; UAS-GFP/TM6B. Control clones were obtained by mating wild type males with females of w`, hsFLP; act>w`>Gal4; UAS-GFP/TM6B. As shown in Figure 5-6, p-Akt staining was increased in Yki overexpressing clones but not in GFP overexpressing control clones.

Figure 5-6: Akt activity is enhanced in Yki overexpressing clones. (D) Yki overexpressing clones were generated by “Flip-out” technique and positively labeled with GFP. (E, F) Ectopic expression of Yki elevates the p-Akt level. (A-C) No change of p-
Akt staining is seen in GFP overexpressing control clones.

5.3 Akt Protein Level Is Negatively Regulated by Hippo Signaling

5.3.1 Akt Protein Level Is Decreased in Regions Expressing Constitutively Active Mats

The regulation of p-Akt level by Hippo signaling could be due to the effect on total Akt protein level or post-translational modification. To test the first possibility, total Akt protein level was examined in the third instar larvae wing discs using anti-Akt antibody (Cell Signaling Technology, Cat. No. 4691).

Constitutively active Myr-mats-GFP was expressed in the posterior region of wing imaginal discs as described before. Total Akt staining was downregulated in Myr-mats-GFP overexpressing region but not in GFP overexpressing area (Figure 5-7).
Figure 5-7: Total Akt protein is downregulated in regions expressing constitutively active Mats. (E-H) Overexpression of Myr-mats-GFP in the posterior region of larvae wing discs (GFP positive) suppresses the total Akt protein level. (A-D) Overexpression of GFP does not affect Akt protein level.

5.3.2 Akt Protein Level Is Increased in Loss of mats, Loss of hpo, or Yki Overexpressing Clones

Generation of loss of mats, loss of hpo, Yki overexpressing or control clones was described before. Loss of function of mats or hpo led to the upregulation of Akt protein level, which was not seen in wild type control clones (Figure 5-8). Consistently, gain of function of Yki but not GFP in clones of cells enhanced the staining of total Akt protein (Figure 5-9).
Figure 5-8: Akt protein level is increased in loss of *mats* or loss of *hpo* clones. (D, G) Loss of *mats* and loss of *hpo* clones were generated by MARCM and positively labeled with GFP. (E, F, H, I) Staining of Akt protein is upregulated in clones of cells mutant for *mats* or *hpo*. (A-C) Akt protein level is not affected in control clones.
Figure 5-9: Akt protein level is elevated in Yki overexpressing clones. (D) Yki overexpressing clones were generated by FLPout technique and positively labeled with GFP. (E, F) Clones of cell overexpressing Yki shows elevated level of total Akt protein. (A-C) Overexpression of GFP does not change Akt protein level.

5.4 Transcription of akt Is Negatively Regulated by Hippo Signaling

Akt activity and protein level have been shown to be down-regulated by Hippo signaling. However, the regulatory mechanism by which Hippo signaling affects Akt molecule is still elusive. It is known that Hippo signaling converges to Yki/Sd to regulate target gene transcription. To examine whether akt is also transcriptionally regulated by Hippo signaling, whole mount in-situ hybridization was performed to evaluate the mRNA level of akt in clones of cells mutant for Hippo signaling in developing fly wing discs.
Loss of *mats* clones were generated by MARCM and positively marked by GFP. GFP fluorescence signal was not stable during the process of in-situ hybridization. Therefore, rabbit anti-GFP antibody (Sigma, Cat. No. G1544) combined with anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen, Molecular Probes) was used. *akt* mRNA was visualized with rhodamine-conjugated anti-DIG antibody (Roche, Cat. No. 11207750910). Images were collected with FluoView FV300 confocal microscope. As shown in Figure 5-10, loss of *mats* clones but not control clones exhibited elevation of *akt* mRNA level.

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**Figure 5-10:** Loss of *mats* function results in an increase of *akt* mRNA level. (D) Loss of *mats* clones were generated by MARCM and positively labeled with GFP. (E, F) *akt* mRNA level is increased within the loss of *mats* clone region. (A-C) *akt* transcription is not affected in control clones (GFP positive).

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Yki was ectopically expressed in the posterior region of wing imaginal discs driven by *engrailed-Gal4* (Figure 5-11 D). *Engrailed-Gal4* driven expression of GFP was functioning as control (Figure 5-11 A). Clones of cells overexpressing Yki or GFP were generated as described before (Figure 5-11 J, G). Overexpression of Yki either in
posterior region of wing discs (Figure 5-11 E, F) or in clones of cells (Figure 5-11 K, L) resulted in increase of akt mRNA level, which was not seen in control area (Figure 5-11 B, C, H, I).
Figure 5-11: Yki up-regulates akt transcription. (D) Yki was overexpressed in the posterior region of wing imaginal discs (to the right) by en-Gal4. (E, F) akt mRNA level is higher than the wild type anterior region. (A-C) akt mRNA level is not changed by GFP overexpression. (J) Clones of cells overexpressing Yki were generated by MARCM and positively marked by GFP. (K, L) Gain of Yki increased the akt mRNA level. (G-I) akt mRNA level is not changed in control clones.

5.5 P-4E-BP Level Is Down-regulated by Hippo Signaling

Eukaryotic initiation factor 4E binding protein (4E-BP) is a downstream component of Akt signaling, which plays an important role in cap-dependent translation of mRNAs. Only one 4E-BP protein exists in Drosophila, but there are three 4E-BP members in mammals, 4E-BP1, 4E-BP2, and 4E-BP3 (Pause et al., 1994; Poulin et al., 1998). In the unphosphorylated state, 4E-BP binds to eukaryotic initiation factor 4E (eIF4E) and blocks the translation initiation. Upon phosphorylation at multiple sites by PI3K/Akt signaling and TOR complex, 4E-BP is disassociated from eIF4E and cap-dependent translation is activated (Brunn et al., 1997; Gingras et al., 1999; Gingras et al., 1998). Therefore, phosphorylation of 4E-BP serves as a sensitive readout for monitoring Akt signaling activity.

To confirm the crosstalk between Hippo signaling and Akt signaling, the influence of Hippo signaling on the level of p-4E-BP was examined in developing fly wing discs. As mentioned above, 4E-BP protein is phosphorylated at multiple sites. The
TOR-mediated phosphorylation of 4E-BP at Thr-37 and Thr-46 is an early event necessary for subsequent phosphorylation and is present in all phosphorylated 4E-BP isoforms (Gingras et al., 1999). Therefore, p-4E-BP1(Thr37/46) antibody (Cell Signaling Technology, Cat. No. 2855) was used to detect the protein level of phosphorylated 4E-BP protein in Drosophila tissue (Species cross-reactivity has been demonstrated by Cell Signaling Technology).

Yki overexpressing clones, loss of mats clones and the control clones were generated as described before. Third instar larvae wing discs were dissected for analysis. Staining pattern of p-4E-BP revealed both cytoplasm and nuclear localization of target protein (Data sheet from Cell Signaling Technology. Figure 5-12 A-C). Ectopic expression of Yki increased the amount of nuclear-localized p-4E-BP, illustrated by the enhanced dot-like staining pattern, which was rarely observed in surrounding wild-type area (Figure 5-12 D-I).
Figure 5-12: P-4E-BP level is upregulated by Yki. (D) Yki overexpressing clones were generated by FLPout technique and positively marked with GFP. (E, F) Dot-like staining of p-4E-BP is enhanced in Yki overexpressing clones. (G-I) Higher magnification images of the same wing discs of (D-F). (A-C) Control clones overexpressing GFP reveals the normal expression pattern of p-4E-BP.

Similarly to Yki overexpressing clones, loss of mats function elevated the overall cytoplasmic level of p-4E-BP and also led to the accumulation of nuclear localized p-4E-BP protein (Figure 5-13 D-I).
Figure 5-13: Loss of mats function stimulates p-4E-BP level. (D) Loss of mats clones were generated by MARCM and positively labeled with GFP. (E, F) P-4E-BP staining is upregulated in clones of cells mutant for mats but not changed in control clones (A-C). (G-I) Magnified view of the framed area in (F).
5.6 Pten Protein Level Is Not Down-regulated by Loss of mats Function

PTEN is an important tumor suppressor gene, whose mutations were found in a wide range of human cancers (Ali et al., 1999). PTEN functions as a lipid phosphatase to dephosphorylate PIP3, thereby blocking the activation of Akt (Maehama and Dixon, 1999). PTEN-mediated regulation of Akt signaling plays an important role in cell proliferation and apoptosis. In addition, PTEN inhibits TOR metabolic pathway to affect individual cell size and organ size (Sansal and Sellers, 2004).

Considering that both PTEN and Mats proteins are tumor suppressors, loss of mats function might affect Pten expression and/or activity. To examine this possibility, two kinds of anti-Pten antibodies were used to detect Pten protein level in loss of mats clones. The first anti-PTEN antibody was obtained from ABGENT (Cat. No. AP8436a). A synthetic peptide of 21 amino acids from N-terminal region of human PTEN was used as antigen, which shares 71% identity with the sequence fragment from Drosophila Pten. The second antibody used was generated against Drosophila Pten by Dr. Vivian Budnik’s lab. As shown in Figure 5-14, however, loss of mats function did not decrease Pten protein level as hypothesized, instead, some of the mats−/− clones exhibited an increase of Pten staining.

Regulation of PTEN protein includes but is not limited to transcriptional regulation, protein-protein interactions and phosphorylation (Leslie and Downes, 2004). C-terminus phosphorylation of PTEN by casein kinase II (CK2) decreases its activity whereas stabilizing PTEN protein. Therefore, protein level and activity of Pten could be modulated through independent mechanisms. Examination of the lipid phosphatase activity of Pten in loss of mats context will help to address this question. On the other hand, since Pten protein level is upregulated by loss of mats, it will be interesting to see whether pten gene transcription is targeted by Hippo signaling, as a negative feedback like ex.
Figure 5-14: Loss of *mats* function does not suppress Pten protein level. (A, D) Loss of *mats* clones were generated by MARCM and positively marked by GFP. (B, C) *Drosophila* Pten was detected with anti-PTEN (human) antibody. (E, F) Anti-dPten (*Drosophila*) antibody was used. No downregulation of Pten protein is seen in loss of *mats* clones, instead, some clones show the upregulation of Pten protein (arrows).

5.7 Subcellular Localization of tGPH Is Influenced by Yki Overexpression

As mentioned above, Akt is negatively regulated by Hippo signaling in the transcription level, protein level and activity, so is the phosphorylation of 4E-BP protein. Activation of Akt is known to be dependent on the plasma membrane recruitment by binding to PIP3 and additional phosphorylation by PDK1 and TOR kinase. To investigate whether the regulation of Akt activity by Hippo signaling is through PI3K or other further
upstream components of Akt pathway, an *in vivo* reporter for PI3K activity, tGPH, was used.

*tGPH* is a recombinant gene generated by fusing GFP to the PH domain, whose expression is under the control of *Drosophila* β-tubulin promoter (Britton et al., 2002). PH domain is phosphoinositide-interacting domain, which is found in several proteins of Akt pathway, such as Akt and PDK. Upon activation of PI3K, PIP3 is accumulated at the plasma membrane and recruits these proteins to the membrane via PH-PI interaction. Therefore, tGPH, the PH domain-containing fusion protein, is able to serve as *in vivo* reporter for PI3K activity based on its subcellular localization status. Transgenic flies ubiquitously expressing *tGPH* gene were obtained from Bloomington Drosophila Stock Center. In addition, transgenic flies carrying UAS- Pi3k.CAAX (a constitutively active form of Pi3k) or UAS-Inr.exel (wild type Inr) were also obtained from the Drosophila Stock Center.

Overexpression of Pi3k, Inr or Yki was driven by *en-Gal4*. Third instar larvae wing discs were used for analysis. In the posterior region of wing discs expressing Pi3k or Inr, the overall tGPH protein level was upregulated (Figure 5-15 A-F), which is consistent with the finding from Edgar’s group (Britton et al., 2002). Similarly, overexpression of Yki driven by *en-Gal4* also increased the overall tGPH protein.
Figure 5-15: Expression of Yki increases tGPH protein level. (A, D, G) Overexpression of Pi3k, Inr or Yki was driven by en-Gal4 in posterior region of wing imaginal discs (to the right as arrows point). (B, C, E, F) As positive controls, ectopic expression of an active form of Pi3k or wild type Inr upregulates the overall protein level of tGPH (upregulation level is milder in en>Inr). (H, I) Overexpression of Yki significantly increases the tGPH protein level. The objective magnification is 20X.

To study the subcellular localization of tGPH, 60 X objective magnification combined with zoom X 2 was used. In the anterior wild type region of wing discs, tGPH signal was observed in cell membrane, cytoplasm and nucleus (Figure 5-16 C, G, K, to
the left of dashed lines). Overexpression of Pi3k or Inr resulted in the increase of membrane localization of tGPH signal in the posterior region of wing discs (Figure 5-16 C, G) or (Britton et al., 2002). Staining of E-cadherin was used as membrane marker (Figure 5-16 B, F, J). This observation validates tGPH as *in vivo* reporter for Inr/Pi3k activity. Next, the effect of Yki overexpression on tGPH localization was examined and the similar result was seen as the membrane localization of tGPH was enhanced (Figure 5-16 K, L). It indicates that Yki is able to regulate Akt signaling by modulating the signal input upstream to PIP3.

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**Figure 5-16**: Expression of Yki increases membrane localization of tGPH. (A, E, I) Overexpression of Pi3k, Inr or Yki was driven by *en-Gal4* in posterior region of wing imaginal discs (to the right). (B, F, J) Staining of E-cadherin outlines the cell membrane. (C, D, G, H, K, L) Membrane localization of tGPH is enhanced in the posterior region (to
the right of dashed lines) expressiong Pi3k, Inr or Yki compared to the anterior wild type region. Images were taken at 60X objective magnification with zoom X 2.

5.8 Epistasis Analysis of Hippo and Akt Signaling Components

5.8.1 Introduction

Introduction of the yeast FLP/FRT system into Drosophila led to the development of a powerful genetic mosaic analysis method called MARCM (Golic and Lindquist, 1989; Lee and Luo, 1999) and (Figure 5-2 for the diagram of MARCM). In this method, the mutant gene of interest and a dominant repressor of cell marker are placed in trans on the homologous chromosome distal to the FRT sites. Upon the expression of FLP recombinase by heat shock, mitotic recombination occurs between the two homologous FRT sites, producing two daughter cells either carrying homozygous mutant gene or homozygous repressor gene. These two daughter cells undergo rounds of cell divisions and generate patches of cells with identical homozygous genotype in the otherwise heterozygous genetic background. Due to the loss of repressor gene, clones mutant for the target gene are positively labeled with the marker (i.e. green fluorescence protein, GFP).

When additional UAS-transgene is combined into this genetic background, this system allows the study of the collaborative effect of gain of gene A function in loss of gene B mutant clones. That is how the epistasis analysis of Hippo and Akt signaling components was carried out.

5.8.2 Effectiveness Test of Pi3kRNAi and AktRNAi Lines

UAS-Pi3kRNAi and UAS-AktRNAi lines were obtained from Vienna Drosophila RNAi Center (VDRC). Overexpression of Pi3kRNAi driven by en-Gal4 reduced the
adult wing size by 17% (Figure 5-17. t-Test, P<0.05). Ectopic expression of AktRNAi by en-Gal4 was lethal with 98% penetrance (n=43) and resulted in a larval growth arrest. It indicates that both RNAi lines are physiologically active at the organismal level.

Figure 5-17: Overexpression of Pi3kRNAi significantly reduces adult wing size. (A) Overexpression of GFP by en-Gal4 was used as control. (B) Overexpression of Pi3kRNAi by en-Gal4 resulted in a dramatic decrease in size of the whole wing (compared to the white dashed area) especially of the posterior region (the region underneath the red dashed line). (C) Fly wings overexpressing Pi3kRNAi (n=5) are 17% smaller than control wings (n=21). Error bar represents standard deviation. t-Test, P<0.05.

At the cellular level, Pi3k is known to activate Akt protein indirectly by converting PIP2 to PIP3. Therefore, the activity of Akt was monitored by p-Akt staining in clones of cells overexpressing Pi3kRNAi transgene. Pi3kRNAi overexpressing clones
were generated by crossing males of \( w^+; UAS-pi3kRNAi; FRT82B P[w^+]/S.T \) with females of \( y^{w}, hsFLP, UAS-GFP; tub-Gal4, FRT82B tub-Gal80/TM6B \). At 40X objective magnification, a decrease of p-Akt staining was seen within the clones (Figure 5-18 D-F). Pi3kRNAi overexpressing clones were small in size, which made it hard to clearly examine the difference of p-Akt level. Therefore, the clones overexpressing Pi3kRNAi in loss of \( mats \) background were generated. Males of \( w^+; UAS-pi3kRNAi; FRT82B mats^{235}/S.T \) were crossed with females of \( y^{w}, hsFLP, UAS-GFP; tub-Gal4, FRT82B tub-Gal80/TM6B \). Within the GFP positively labeled clones, p-Akt level was significantly decreased, which supported the high efficiency of Pi3kRNAi (Figure 5-18 G-I).
Figure 5-18: Expression of Pi3kRNAi decreases Akt activity. (A, D, G) Clones overexpressing Pi3kRNAi with (G) or without (A, D) loss of mats function were generated by MARCM and positively marked with GFP. Staining of p-Akt is downregulated by Pi3kRNAi overexpression, which is shown at 20X (B, C) and 40X (E, F) objective magnification. (H, I) When combined with loss of mats function, Pi3kRNAi overexpressing clones are bigger and display dramatic suppression of p-Akt level. (J-L) As mentioned before, loss of mats function upregulates p-Akt level.

To examine whether AktRNAi does inhibit akt transcription, Akt protein level was tested in AktRNAi overexpressing clones. Clones overexpressing AktRNAi in loss of mats background were generated by mating males of w; UAS-aktRNAi; FRT82B mats^235/S.T. with females of y\(\text{w}\), hsFLP, UAS-GFP; tub-Gal4, FRT82B tub-Gal80/TM6B. These clones were large in size and exhibited distinct downregulation of Akt protein level (Figure 5-19). Therefore, AktRNAi functions in downregulating Akt protein level, presumably due to its suppression on akt mRNA.
Figure **5-19**: Expression of AktRNAi downregulates Akt protein level. (A) Clones overexpressing AktRNAi with loss of *mats* function were generated by MARCM and positively marked with GFP. (B, C) Akt protein level is severely suppressed within the clones.

### 5.8.3 Genetic Interaction between *akt* and *mats*

Both Akt signaling and Hippo signaling are involved in growth control by regulating similar biological processes, such as cell proliferation, cell growth and apoptosis (Oldham and Hafen, 2003; Pan, 2007). In this work, loss of *mats* function has been shown to be able to upregulate Akt protein level as well as its activity. This raised the curiosity to see whether *akt* and *mats* functionally interact. One way to study the functional interaction between two genes is to manipulate one gene dosage and to examine its effect on the phenotype of another gene.

UAS-Akt transgenic flies were obtained from Bloomington Drosophila Stock Center. Overexpression of Akt by *en-Gal4* increased the overall wing size by 12% (Figure **5-20** B and D). Loss of one *mats* allele sensitized the growth promoting activity of Akt, where the fly wings were 25% larger than control wings (Figure **5-20** C and D). This is supportive of the genetic interaction between *akt* and *mats* genes.
Figure 5-20: *mats* and *akt* genetically interact. (A) Control fly wing overexpressing GFP. (B) Akt-overexpressing wing is larger than control wing, most prominently in the posterior region. (C) Half reduction of *mats* gene dosage enhanced the wing overgrowth phenotype associated with Akt overexpression. (D) Fly wings overexpressing Akt by *en-Gal4* (n=35) are 12% larger than control wings (n=21). Loss of one allele of *mats* (n=13) increased the wing size enlargement to 25%. Error bar refers to standard deviation.

To examine the expression level of Akt in en>Akt transgenic flies, staining of Akt protein was performed in the third instar larvae wing discs. Strong Akt staining was seen in the posterior region expressing UAS-Akt transgene (Figure 5-21).
Figure 5-21: en-Gal4 drives ectopic Akt expression in wing imaginal discs. Overexpression of Akt was driven by en-Gal4 with (C, D) or without (A, B) heterozygous mats mutant allele. Akt expression (red) is both dramatically induced in the posterior region of wing discs (to the right).

5.8.4 Epistasis Analysis of pi3k and mats

Epistasis analysis can be used to order the function of two genes when both genes are controlling a common biological process (Huang and Sternberg, 2006). Phenotype of one mutant gene is able to be masked by phenotype of its epistatic mutant gene. When two genes of interest cause opposite phenotypes, double mutants can be constructed to determine their epistatic relationship.

In this work, overexpression of Pi3kRNAi was induced in loss of mats clones by MARCM. If the overgrowth phenotype of mats mutant could be overridden by Pi3kRNAi expression, pi3k is epistatic, or downstream, to mats. However, that was not what we observed. Compared to control clones (Figure 5-22 A, A’), overexpression of Pi3kRNAi alone produced smaller clones (Figure 5-22 B, B’), consistent with the growth-promoting activity of Pi3k. As reported before, loss of mats function gained growth advantage and led to distinguished clonal expansion (Figure 5-22 D, D’). Overexpression of Pi3kRNAi
in loss of *mats* clones generated clones more similar to loss of *mats* alone, where the GFP-positive area occupied most of the wing discs.

Considering Pi3kRNAi instead of null *pi3k* mutant was used, there might be some leaking activity of Pi3k, which confers the signal transduction of Mats. It is also likely that the regulation of Mats on Akt pathway occurs at further downstream component, for example, Akt. Therefore, the genetic relationship of *akt* and *mats* was next analyzed, which would be mentioned in the following chapter.

Figure 5-22: Clones overexpressing Pi3kRNAi and mutant for *mats* are more similar to loss of *mats* alone. Clones were generated by MARCM and positively marked by GFP. (A, A’) Control clones. (B, B’) Clones overexpressing Pi3kRNAi alone are somewhat smaller than control clones. (C, C’) Overexpression of Pi3kRNAi in loss of *mats* clones does not affect *mats* ¬¬-associated clonal expansion activity. (D, D’) Loss of *mats* clones show growth advantage over control clones.

Although the clonal expansion activity of *mats* mutant is not dependent on Pi3k, Figure 5-18 indicated that upregulation of p-Akt by loss of *mats* was overridden by
Pi3kRNAi overexpression. Therefore, in terms of the regulation of Akt activity, pi3k is epistatic to mats.

Since Akt activity is blocked in double mutant for pi3k and mats, the question is what might be the mediator for the growth-promoting activity as seen in the large double mutant clones (Figure 5-22 C, C’). To examine whether this mediator could be some other downstream component of Pi3k/Akt pathway, p-4E-BP was used as read out for the effect of pi3k and mats double mutant on Akt signaling. Overexpression of Pi3k RNAi alone slightly reduced the level of p-4E-BP (Figure 5-23 A-C). This effect, however, was masked by loss of mats function since the double mutant clones expressing Pi3kRNAi in mats mutant background showed similar phenotype as loss of mats alone (Figure 5-23 D-F compared to Figure 5-13 D-I). This is supportive of the additional Mats-regulating target protein downstream to Pi3k.

Figure 5-23: Overexpression of Pi3kRNAi in mats mutant clones upregulates p-4E-BP, similar to loss of mats alone. All clones were generated by MARCM and positively marked by GFP. (A-C) Overexpression of Pi3kRNAi alone results in a little decrease of p-4E-BP staining (as arrow points to). (D-F) Overexpression of Pi3kRNAi in loss of mats
clones accumulates dot-like signal of p-4E-BP, which is similar to loss of mats function alone (Figure 5-13 D-I).

5.8.5 Epistasis Analysis of akt and mats

Similar phenotypic analysis was used to dissect the epistatic relationship of akt and mats. Double mutant clones overexpressing AktRNAi and mutant for mats were generated by MARCM and positively marked with GFP. Similar to Pi3kRNAi overexpressing clones, overexpression of AktRNAi produced smaller and less number of clones than control (Figure 5-24 compare B, B’ with A, A’). As expected, loss of mats clones were large in size (Figure 5-24 D, D’). Overexpression of AktRNAi in loss of mats clones, however, did not mask the clonal expansion activity of mats /-, producing clones similar to single mats mutant (Figure 5-24 C, C’).
Figure 5-24: Clones overexpressing AktRNAi and mutant for *mats* are more similar to loss of *mats* clones. All clones were generated by MARCM and positively marked by GFP. (A, A’) Control clones. (B, B’) Overexpression of AktRNAi in control clones shows growth disadvantage and produces smaller and fewer GFP-positive patches. (C, C’) Clones overexpressing AktRNAi plus loss of *mats* function are closer to *mats*−/− clones (D, D’) in both clone size and clone number.

Akt is known to phosphorylate 4E-BP protein via rapamycin-sensitive TOR complex (Gingras et al., 1998). It was described earlier here that loss of *mats* caused an increase of p-4E-BP protein level. Next, staining of p-4E-BP was performed in *akt* and *mats* double mutant clones to see whether the upregulation of p-4E-BP by loss of *mats* is dependent on *akt*. As mentioned before, wild-type control clones did not affect p-4E-BP staining (Figure 5-25 A-C) whereas loss of *mats* function increased the overall protein level and enhanced the nuclear staining of p-4E-BP (Figure 5-25 G-I). Reduction of *akt* function by overexpressing AktRNAi appeared to downregulate the overall staining of p-4E-BP and decreased the dot-like signal (Figure 5-25 D-F). Overexpression of AktRNAi
in loss of *mats* clones, however, did not override the upregulation of p-4E-BP by *mats* mutant, showing similar phenotype as loss of *mats* alone (Figure 5-25 J-L).

Figure 5-25: Overexpression of AktRNAi in *mats* mutant clones does not block upregulation of p-4E-BP by loss of *mats*. All clones were generated by MARCM and positively marked by GFP. (A-C) Normal staining pattern of p-4E-BP in control clones. (G-I) Similar to Figure 5-13 (D-I), upregulation of p-4E-BP is observed within loss of *mats* clones. (D-F) Clones expressing AktRNAi show a little decrease of p-4E-BP
staining and less nuclear-localized signal (as arrow points to). (J-L) Double mutant clones overexpressing AktRNAi and mutant for mats increase overall p-4E-BP staining and accumulate dot-like signal, similar to loss of mats clones.

5.9 Epistasis Analysis of mats and yki

5.9.1 yki Is Functionally Epistatic to mats

Yki has been well known as the transcriptional coactivator to regulate target gene expression of Hippo pathway (Huang et al., 2005). Upon phosphorylation by Wts kinase, Yki is excluded from the nuclear and its activity to regulate gene transcription is inhibited. Mats was identified as activating subunit of Wts kinase, which suggests Mats acting upstream to Wts (Lai et al., 2005). However, at developing stage, double mutant clones for loss of mats and gain of wts are similar to loss of mats alone (data of Shimizu, 2006). This is supportive of mats being downstream to wts. Therefore, mats and wts might function in a loop fashion instead of a linear way.

To further linearize Hippo pathway, the epistatic relationship of mats and yki was analyzed. YkiRNAi was overexpressed in loss of mats clones by MARCM and the clones were positively labeled with GFP. Overexpression of YkiRNAi alone generated very few clones, which were even smaller in size than control clones (Figure 5-26 B, B’ compared to A, A’). Double mutant clones for loss of mats and yki were similar to loss of yki alone (Figure 5-26 C, C’ compared to B, B’). The overgrowth phenotype induced by loss of mats was masked by the reduction of Yki function, which supported yki as epistatic molecule to mats (Figure 5-26 C, C’ compared to D, D’).
Figure 5-26: Clones overexpressing YkiRNAi and mutant for mats are more similar to YkiRNAi overexpressing clones. Clones were generated by MARCM and positively marked by GFP. (A, A’) Control clones generated by 2 hr heat shock at 1 day AEL. (B, B’) YkiRNAi overexpressing clones are similar to control clones. (C, C’) YkiRNAi overexpression overrides the growth advantage associated with loss of mats function (D, D’). The clones are like YkiRNAi overexpression alone in both clone size and clone number.

5.9.2 mats Mutant-Associated Upregulation of Akt is Dependent on yki

As described in previous chapter, loss of mats or gain of yki function increased Akt protein level (Figure 5-8, 5-9). Although mats and yki have been shown to act in Hippo pathway to control growth, it is not known whether their function in regulating Akt molecule is related. The epistatic relationship of yki and mats was just clarified in
terms of their clonal expansion activity. It would be interesting to see whether the specific function of mats mutant to positively regulate Akt protein is dependent on yki.

To address this question, Akt staining was performed in double mutant clones expressing YkiRNAi and lacking mats function. As shown in Figure 5-27 (A-C), overexpression of YkiRNAi alone dramatically reduced Akt protein level, which is consistent with the positive regulation of Akt protein by ectopic Yki expression (Figure 5-9). Loss of mats function did not reverse this phenotype, where the double mutant clones showed similar regulation of Akt as loss of yki alone (Figure 5-27 D-F compare to A-C). This is supportive of yki acting downstream to mats to positively regulate Akt protein.

**Figure 5-27:** YkiRNAi blocks the Akt upregulation induced by loss of mats. Clones were generated by MARCM and positively labeled with GFP. (A-C) Reduction of yki function by YkiRNAi overexpression significantly decreased Akt protein level. (D-F) Overexpression of YkiRNAi in loss of mats clones still shows reduction of Akt signal.
5.10 Chapter Summary

In this project, the intervening relationship between Hippo and Akt signaling pathways was examined. Akt, the core regulator of cell growth, proliferation and cell death, acts downstream of InR, Pi3k and PDK to transduce growth regulatory signal to TOR complex (Hietakangas and Cohen, 2009). Due to its critical role in Akt signaling as well as the availability of appropriate research reagents, Akt was originally tested for its putative role as a Hippo signaling target. MARCM or FLPout techniques were used to generate mosaic clones homozygously mutant for Hippo pathway components. The results showed that Hippo signaling negatively regulates Akt activity as well as its mRNA and protein levels. Yki overexpression is sufficient to increase the production of akt mRNA as well as Akt protein, suggesting that akt might be another target gene of Hippo signaling. Higher level of akt mRNA is expected to increase Akt protein synthesis. Moreover, elevated level of Akt protein could be due to the increased stability. The enhanced activity of Akt in loss of mats clones, detected by its phosphorylation states, is dependent on Pi3k since overexpression of Pi3kRNAi effectively overrode this phenotype. Therefore, the interaction between Hippo and Akt signaling does not appear to occur only through Akt. Instead, the upstream components of Akt signaling could be targeted by Hippo signaling as well.

Consistent with the negative regulation of Akt by Hippo signaling, the phosphorylation state of 4E-BP protein was also suppressed by Hippo pathway. 4E-BP and S6K are two best known effectors of Akt signaling as phosphorylation substrates of TOR kinase (Hay and Sonenberg, 2004). However, Radimerski et al. reported that phosphorylation of Drosophila S6K is independent of Akt/Pi3k, but requires PDK (Radimerski et al., 2002). Considering the controversial interpretations about the regulation of S6K by Akt kinase (Lizcano et al., 2003; Long et al., 2005; Sarbassov et al., 2005), p-4E-BP instead of p-S6K was used as the readout of Akt activity in this study. The level of P-4E-BP was increased in both loss of mats and Yki-overexpressing clones.

PTEN is a well known tumor suppressor in mammals, whose loss of function mutation was found in human cancers with a frequency comparable to p53 (Cantley and
Neel, 1999; Simpson and Parsons, 2001). In the cellular context, Pten functions as a negative regulator of Akt signaling upstream of PIP3 (Cantley and Neel, 1999). Staining of Pten protein in loss of mats clones, however, did not show downregulation as expected. Instead, some loss of mats clones slightly increased Pten protein level. It indicates that stimulation of Akt activity by loss of mats function is not via the relief of the inhibition effect from Pten. To clarify the precise effects of Hippo signaling in Pten protein, clones mutant for other Hippo pathway components need to be analyzed.

Effect of Hippo signaling on upstream components of Akt pathway was also examined by using a constitutively expressed GFP-PH domain fusion gene (tGPH) as reporter. Overexpression of Yki driven by en-Gal4 induced membrane accumulation of tGPH signal, in a way similar to ectopic Inr or Pi3k expression. It suggests that, in addition to its influence on Akt, Hippo signaling might also affect Akt signaling input from extracellular environment or other components upstream of lipid phosphorylation. Our results from this study are summarized in Figure 5-28.

Figure 5-28: Summary of the interplay between Hippo and Akt signaling pathways. Arrows represent activation effects. Bar-headed lines represent inhibition effects. Single broken black arrows represent the Hippo signaling input. Single broken colored lines (red
for inhibition, blue for activation) refer to indirect regulation, where the intermediate components are not shown. Double lines refer to the influence of Hippo signaling on the Akt pathway.

Adult wing phenotype supported the genetic interaction between \textit{akt} and \textit{mats} genes, which prompted the epistasis analysis. Clones double mutant for \textit{pi3k} and \textit{mats} were overgrown to the extent similar to \textit{mats} mutant alone. Similarly, \textit{akt} and \textit{mats} double mutations also led to clonal overgrowth, which was closer to although milder than \textit{mats} mutant clones. When P-4E-BP was used as readout, neither loss of \textit{pi3k} or loss of \textit{akt} blocked the induction of P-4E-BP in \textit{mats} mutant clones. It indicates that \textit{akt} or \textit{pi3k} is not crucial, although needed to some extent in the case of \textit{akt}, for \textit{mats} mutant-induced clonal expansion or 4E-BP phosphorylation. Therefore, there might be some other component(s) downstream to Akt, such as Tsc1/2 or Tor, to mediate the upregulation of P-4E-BP and tissue overgrowth in the absence of Hippo signaling.
Chapter 6
Conclusions and Perspectives

6.1 Conclusions

Within this thesis, I described the identification of Sd as the new component of Hippo signaling pathway, acting as transcription factor in the complex with Yki to regulate tissue growth. Physical interaction between Sd and Yki was demonstrated by \textit{in vitro} pull-down assay, which also proved the critical role of Serine 97 of Yki in mediating Sd binding. Analysis of adult eye and wing phenotypes supported the functional significance of Sd binding in Yki/YAP-dependent tissue overgrowth. Loss of Sd binding compromised Yki/YAP activity in inducing extra interommatidial cells as well as clonal expansion. A series of experiments strongly established the genetic interaction between \textit{sd} and \textit{yki}. In the mammalian system, Zhao et al. showed that TEADs, orthologs of \textit{Drosophila} Sd, are important to mediate YAP-dependent gene expression. TEAD-binding is required for YAP-induced cell growth, anchorage-independent growth, and epithelial-mesenchymal transition (EMT) (Zhao et al., 2008). Three other laboratories have also reported that Sd functions as a Yki partner to mediate the growth control activity of Hippo pathway via regulating the target gene expression, which is completely consistent with our findings (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b).

Next, we examined the functional role of the two WW domains of Yki/YAP proteins. WW domains turned out to play a positive role in mediating Yki/YAP-dependent growth control although there was speculation that WW domain is required for Wts-mediated inhibition of Yki activity (Huang et al., 2005). Mutations of WW domains dramatically suppressed the potential of Yki/YAP in promoting adult eye or wing tissue overgrowth, inducing extra cell numbers, or stimulating clonal expansion. The work in mammalian cultured cells from Guan’s lab showed that the functions of YAP in inducing
target gene expression, stimulating cell proliferation and promoting oncogenic transformation are mediated by WW domains in a way independent of TEAD (Zhao et al., 2009). Therefore, some other transcription factors must exist to cooperate with Yki/YAP to regulate target gene expression and exert biological functions.

In my third project, the crosstalk between Hippo and Akt signaling pathways was explored. Akt was shown to be negatively regulated by Hippo signaling at the levels of mRNA, protein and activity. Consistently, the phosphorylation level of 4E-BP protein, an important downstream component of Akt signaling pathway, was also downregulated by Hippo signaling. \textit{yki} proved to be epistatic to the loss of \textit{mats}-induced upregulation of Akt protein level. Loss of Hippo signaling, however, did not show obvious influence on Pten protein. Overexpression of Yki in posterior region of wing discs induced visible although not dramatic membrane accumulation of tGPH similarly to the effect of ectopic Inr or Pi3k overexpression. It brings up the possibility that Hippo signaling might affect Akt signaling via extracellular input or upstream to lipid phosphorylation, although Akt has been shown to be a regulatory target of Hippo signaling. Adult wing phenotype supported the genetic interaction between \textit{akt} and \textit{mats} genes. However, epistatic analysis showed that \textit{akt} or \textit{pi3k} is not crucial for \textit{mats} mutant-induced clonal expansion or 4E-BP phosphorylation. Considering the high efficiency of both \textit{akt} and \textit{pi3k} RNAi lines, the dispensability of \textit{akt} or \textit{pi3k} in loss of \textit{mats}-related functions indicates that other component(s) downstream to Akt, such as Tsc1/2 or Tor, might be the regulated targets of Hippo signaling as well (Figure 6-1).
Figure 6-1: Model of the possible crosstalk between Hippo and Akt signaling pathways. Arrows represent activation effects. Bar-headed lines represent inhibition effects. Single lines are used to connect Akt pathway components, whereas double lines refer to the influence of Hippo signaling on the Akt pathway. Broken double lines with question marks suggest the putative relationships to be examined. Single broken arrows represent the Hippo signaling input.
6.2 Perspectives and Future Directions

6.2.1 Identification of Other Transcription Factors of Yki/YAP

In spite of rapid progresses in the field, many key questions remain to be answered. First of all, the Hippo signaling pathway is unlikely to be linear. Fat is found to be dispensable for the function of Hippo signaling in *Drosophila* posterior follicle cell maturation (Polesello and Tapon, 2007). Other receptor(s) that act in parallel with Fat may exist to relay the extracellular signal via Mer to Hippo pathway. Secondly, in spite of the well recognized oncogenic properties, YAP has also been shown to convey proapoptotic function via tyrosine phosphorylation by c-Abl in response to DNA damage (Levy et al., 2008). The question is how the different functions of YAP are coordinated under different contexts. In spite of the many unknown aspects of Hippo signaling, the most relevant question to my work described above is the identity of the other transcription factors which cooperate with Yki/YAP to exert their growth promoting functions.

Functional analysis in *Drosophila* as well as in mammalian system has shown that WW domains of Yki/YAP are critical for their growth-promoting activity and the induction of a subset of YAP-dependent target genes (Zhao et al., 2009). Therefore, the PPXY-motif containing transcription factors are possible candidates that mediate the WW domain-dependent functions of Yki/YAP via PPXY-WW domain interaction.

Yeast two-hybrid results can be the starting point to narrow down the candidate transcription factors (Giot et al., 2003). HRE (Hippo responsive element)-reporter system could be used as the readout of Yki transcription activity (Wu et al., 2008; Zhang et al., 2008b). Guan’s lab performed the transcription factor library screening for YAP targets in mammalian system (Zhao et al., 2008). *Drosophila* homologs of the positive hits could be tested as well.
6.2.2 Specific Crosstalk Mechanisms between Hippo and Akt Signaling Pathways

6.2.2.1 Role of akt in mats-dependent Growth Control

In our study, it has been shown that Akt is negatively regulated by Hippo signaling at the levels of akt mRNA, Akt protein, as well as Akt activity. The interaction between Hippo and Akt pathways in mammalian system was also reported by Haber’s group, showing that reduction of mammalian LATS1 levels or YAP overexpression increased AKT phosphorylation in serum-starved MCF10A cells (Zhang et al., 2008a). However, they didn’t observe the increase of total AKT protein levels in either manipulation (Zhang et al., 2008a). Therefore, the regulation of Akt by Hippo signaling in Drosophila is not the same as and might be more complicated than the regulation in mammals. Careful dissection of the regulatory elements of akt gene will help to validate the identity of akt as a transcriptional target of Hippo signaling.

The second question to be answered is whether Akt is crucial in mediating loss-of-mats-induced tissue overgrowth. The epistatic analysis between akt and mats or pi3k and mats was based on the RNAi lines. In spite that the efficiency of each RNAi line has been carefully examined, there is always a concern that the target gene is not completely knocked out. The clones mutant for mats and overexpressing akt RNAi are closer to although a little milder than mats mutant clones, still showing overgrowth compared to wild-type controls. It indicates that loss of mats is sufficient to stimulate clonal overgrowth with reduced if not zero level of Akt. To examine whether akt is completely dispensible for mats-induced growth control, the flies carrying null mutation of akt are needed to perform the epistasis analysis. First of all, the comparision between akt mutant clones and akt RNAi overexpressing clones will help to determine the precise efficiency of akt RNAi. If null akt is able to effectively block the growth-promoting acvitity of mats mutant, akt should be the crucial intervening node between Hippo and Akt signaling. Otherwise, investigation of other candidate genes needs to be carried out.
6.2.2.2 Identification of Other Hippo Signaling Targets in Akt Pathway

Downstream to Akt, Tsc1 and Tsc2 function as negative regulators of Akt signaling. Loss of either tsc function has been shown to lead to a cell-autonomous increase in cell size and proliferation (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). However, its main downstream target, Rheb (Ras homologue enriched in brain), seems to only promote cell growth without affecting cell proliferation (Saucedo et al., 2003; Stocker et al., 2003). To linearize the epistatic relationship between mats and tsc genes, mosaic clones mutant for mats whereas overexpressing tsc could be generated by MARCM technique and overgrowth proteital of the double mutant clones could be compared to clones mutant for mats alone or overexpressing tsc alone. Considering that Tsc1 abd Tsc2 function as a heterodimer, it might be necessary to coexpress both tsc1 and tsc2 for the aboved mentioned epistasis analysis.

Another important downstream player of Akt signaling is TOR. TOR is a serine/threonine kinase, which is negatively regulated by rapamycin-FKBP12 complex (Chung et al., 1992). TOR pathway is responsive to Inr/Akt signaling and other stimuli, such as nutrients and stress. TOR functions as an essential regulator of cell growth and proliferation via modulating protein synthesis, ribosomal biogenesis and cell morphology (Hay and Sonenberg, 2004). S6K and 4E-BP are two well known phosphorylation targets of TOR kinase, which in turn confer the activity of TOR in regulating protein translation and cell size (Hay and Sonenberg, 2004). Akt and TOR are closely related, where TORC2 phosphorylates and activates Akt in a rapamycin-insensitive way and Akt in turn stimulates TORC1 activity indirectly (Brazil and Hemmings, 2001; Sarbassov et al., 2005). Epistatic relationship between mats and tor can be analyzed by generating MARCM clones mutant for these two genes, in a way similar to the analysis of mats and akt. By comparing the double mutant clones with either single mutant clones, the epistatic relationship of mats and tor could be revealed.
References


the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. J Biol Chem 278, 33334-33341.


Appendix

Supplementary Data

A.1 Identification of Candidate Enhancers or Suppressors of wts by Screening of Deficiency Lines

To identify candidates of enhancers or suppressors of wts gene, males of 155 deficiency lines from Drosdel Project (Ryder et al., 2004) were crossed with females of w; ey-Gal4; UAS-wts/S.T.. For control experiment, 176 males were crossed with females of w; ey-Gal4; UAS-wts/S.T..

Table A-1: Summary of Deficiency Lines

<table>
<thead>
<tr>
<th>chromosome</th>
<th>total # of lines</th>
<th># of lost lines</th>
<th># of crossed lines</th>
<th># of positive lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>2nd</td>
<td>51</td>
<td>1</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>3rd</td>
<td>83</td>
<td>3</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>4th</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>155</td>
<td>4</td>
<td>151</td>
<td>10</td>
</tr>
</tbody>
</table>

The progenies of control crosses, which overexpressed wts gene under ey-Gal4 driver, showed smaller eye size with cone shape occasionally (Figure A-1 A). Late pupal lethality was about 10.5% (n=119). The dead pupae showed very similar eye phenotypes as hatched adult flies. For the putative enhancers, the progenies either had even smaller adult eye size or had more severe lethality or both compared to the control crosses. For the putative suppressors, however, the small eye phenotypes or pupal lethality or both were suppressed.
For example, the crosses between \( \text{ey}\times\text{wts} \) and \#8050 showed much higher late pupal lethality and the eyes of dead pupae were extremely small and sometimes there was only one eye left (Figure A-1 B). The surviving adult progenies, however, had similar eye phenotypes as control flies (data not shown). \textit{bantam (ban)} gene is located within the genomic region of \#8050 and has been shown to act downstream of \textit{wts} gene in Hippo signaling pathway (Nolo et al., 2006; Thompson and Cohen, 2006). Therefore, we crossed \( \text{ey}\times\text{wts} \) with a \textit{ban} mutant line and got the similar results as \#8050. The late pupal lethality was about 51% (\( n=68 \)) and the eyes of dead pupae were tiny and many of the pupal flies only had one eye left (Figure A-1 C). The surviving adult flies, however, had similar eye phenotypes as control crosses.

Figure A-1. \#8050 and \textit{ban} show similar enhancement of small eye phenotype associated with \textit{wts} overexpression. A. Control flies of \( w; \text{ey-Gal4} /+; \text{UAS-wts} /+ \) have small adult eyes with irregular shape. B. Dead pupae of \( w; \text{ey-Gal4} /+; \text{UAS-wts} /\#8050 \) have dramatically smaller eyes than control flies. C. Dead pupae of \( w; \text{ey-Gal4} /+; \text{UAS-wts /ban} \) have similar eye phenotypes as B.

The observation from Figure A-1 indicated that screening of deficiency lines is a reliable tool to provide information about the functions of the genes located within the genome gaps. It could be used as a starting point to narrow down the candidate genes with putative functions in Hippo signaling pathway for growth control. The screening results are summarized in Table A-2.
Table A-2: Putative Enhancers and Suppressors of \textit{wts}

<table>
<thead>
<tr>
<th>Function</th>
<th>Def Lines</th>
<th>Genomic Region of Def Lines</th>
<th>Positive(+) or Negative(-)</th>
<th>Genomic Region of Candidate Genes</th>
<th>Some Representative Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enhancers</td>
<td>#8048</td>
<td>61C1 - 61E2</td>
<td>+</td>
<td>61C7 - 61C9</td>
<td>Reg-2, bantam MED30, Rev1 RabX6, CG3279 CG13894 CG13895 emc, etc.</td>
</tr>
<tr>
<td></td>
<td>#8049</td>
<td>61C3 - 62A2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#8050</td>
<td>61C7 - 62A2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#8051</td>
<td>61C9 - 61F7</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#8921</td>
<td>87E3 – 88A4</td>
<td>+</td>
<td>87E3 – 88A4</td>
<td>Ace, Ravus Os22, mth112 yellow-e, B52 Adgf-C, Nsf2 ems, Art9 Orc2, etc. ~ 100 genes</td>
</tr>
<tr>
<td></td>
<td>#8957</td>
<td>86C7 - 86E11</td>
<td>+</td>
<td>86C7 – 86D8</td>
<td>CG14693, SelR Tsp86D, Fdh Sodh-2, Adk3 Ugt86Di etc. ~ 40 genes</td>
</tr>
<tr>
<td></td>
<td>#8968</td>
<td>86D8 – 86E13</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suppressors</td>
<td>#8923</td>
<td>93F14 – 94B5</td>
<td>+</td>
<td>94A2 – 94B4</td>
<td>CG13407, sar1, PSR mats, Nrx-1 CG5278, etc. ~ 80 genes</td>
</tr>
<tr>
<td></td>
<td>#8924</td>
<td>94A2 – 94C4</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#8684</td>
<td>94B4 – 94E6</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>#8038</td>
<td>23B6 – 23D1</td>
<td>+</td>
<td>23B8 – 23D1</td>
<td>Rbp9, Ts, Rrp1, gammaTub23C Ch1, Bem46 Ork alpha4GT1 CG3523 Toc, etc. ~ 30 genes</td>
</tr>
<tr>
<td></td>
<td>#8904</td>
<td>23B8 – 23F6</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### A.2 Drosophila Stock Lines Used

Table A-3: Summary of *Drosophila* Stock Lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Common Tools</strong></td>
<td></td>
</tr>
<tr>
<td>AP-Gal4/Cyo</td>
<td>#3041</td>
</tr>
<tr>
<td>w; GMR-Gal4/SM1; Sb/TM6</td>
<td></td>
</tr>
<tr>
<td>w; Adv/SM1; C5-Gal4/TM6</td>
<td></td>
</tr>
<tr>
<td>yw; tub-Gal4/S.T.</td>
<td></td>
</tr>
<tr>
<td>nb-Gal4(II)</td>
<td></td>
</tr>
<tr>
<td>w; en-Gal4/SM1; Sb/TM6</td>
<td></td>
</tr>
<tr>
<td>AB1-Gal4(III)</td>
<td>#1824 (lost)</td>
</tr>
<tr>
<td>w; ey-Gal4/S.T.</td>
<td></td>
</tr>
<tr>
<td>w</td>
<td>176</td>
</tr>
<tr>
<td>Canton S</td>
<td></td>
</tr>
<tr>
<td>w; Adv/SM1; Sb/TM6</td>
<td></td>
</tr>
<tr>
<td>GMR-Gal4(II)</td>
<td></td>
</tr>
<tr>
<td>en-Gal4(II)</td>
<td>#6356</td>
</tr>
<tr>
<td>w; C5-Gal4(III)</td>
<td></td>
</tr>
<tr>
<td>w; hs-Gal4/Cyo</td>
<td>#2077</td>
</tr>
<tr>
<td><strong>TM3/TM6B</strong></td>
<td></td>
</tr>
<tr>
<td>Elav-Gal4(II)</td>
<td></td>
</tr>
<tr>
<td>Appl-Gal4</td>
<td></td>
</tr>
<tr>
<td>w; gp150³/S.T.</td>
<td></td>
</tr>
<tr>
<td>w, hsFLP; Act&gt;w⁺&gt;Gal4; UAS-GFP/TM6B</td>
<td>Low Temp raising, 31 degree heat shock</td>
</tr>
<tr>
<td>hsflp; AyGal4,UAS-GFP; MKRS/TM6B</td>
<td></td>
</tr>
<tr>
<td>w; fj-lacZ (II)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. Montell (lost)</td>
</tr>
<tr>
<td></td>
<td>K. Irvine</td>
</tr>
<tr>
<td>Genotype</td>
<td>Source</td>
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<tr>
<td>----------</td>
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<tr>
<td>w; ex-lacZ/Cyo</td>
<td>K. Irvine</td>
</tr>
<tr>
<td>w; fj-lacZ,GMR-Gal4; +/S.T.</td>
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<tr>
<td>w; fj-lacZ,GMR-Gal4; +/S.T.</td>
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<tr>
<td>w; fj-lacZ; C5-Gal4/S.T.</td>
<td>3</td>
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<tr>
<td>w; FRT42D/Cyo</td>
<td>M. Rolls</td>
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<tr>
<td>w,FRT18A</td>
<td>#1578</td>
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<tr>
<td>w; FRT82BP[w⁺]</td>
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<tr>
<td>w; Adv/SM1; FRT82B90E/TM6</td>
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<tr>
<td>w; FRT42DP[w⁺]</td>
<td>#1928</td>
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<td>w,ubi-GFP(565T)nls,FRT18A/Fm7a</td>
<td>#5624</td>
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<td>w,arm-lacZ,FRT18A</td>
<td>#7370</td>
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<td>w,FRT19A,tub-Gal4,hsFLP; UAS-MCD8::GFP(II)</td>
<td>#5134</td>
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<td>w; MKRS,hsFLP/TM6B,tb,hu</td>
<td>#279</td>
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<tr>
<td>hsflp122,UAS-GFP; FRT42Dtub-Gal80; tub-Gal4/TM6B</td>
<td>J. Treisman</td>
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<tr>
<td>yw,hsflp,UAS-GFP; tub-Gal4,FRT82Btub-Gal80/TM6B</td>
<td>N. Perrimon</td>
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<td>hsflp; tub-Gal4,FRT82B,tub-Gal80/TM6B</td>
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<tr>
<td>w; Adv/SM1; th⁵c⁸/TM6</td>
<td>diapl-lacZ (lost)</td>
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<tr>
<td>w; Sp/Cyo; 16.4-lacZ</td>
<td>cycE-lacZ</td>
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<tr>
<td>w,en-Gal4; UAS-GFP/S.T.</td>
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<tr>
<td>w; UAS-GFP(II)(nls)</td>
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<td>w; Adv/SM1; UAS-GFP/TM6</td>
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<tr>
<td>hsfIp,act&gt;CD2&gt;Gal4(X)</td>
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<tr>
<td>w; UAS-DsRed (210D)(II)</td>
<td>R. Ordway</td>
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<tr>
<td>w; UAS-DsRed(44A)/TM6B</td>
<td>R. Ordway (bright line)</td>
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<td>w; Adv/SM1; UAS-DsRed(44A)/TM6</td>
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**Hpo, Mats, Wts lines**

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<tr>
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<tr>
<td>w; FRT82Bmats235/TM6B</td>
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<td>Genetic Line</td>
<td>Remarks</td>
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<td>--------------</td>
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<tr>
<td>w; UAS-GFP(nls); FRT82Bmats&lt;sup&gt;94a&lt;/sup&gt;/S.T.</td>
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<td>w; GMR-Gal4; FRT82Bmats&lt;sup&gt;94a&lt;/sup&gt;/S.T.</td>
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<tr>
<td>w; ey-Gal4; FRT82Bmats&lt;sup&gt;94a&lt;/sup&gt;/S.T.</td>
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<tr>
<td>w; en-Gal4; FRT82Bmats&lt;sup&gt;235&lt;/sup&gt;/S.T.</td>
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<tr>
<td>w; UAS-EGFP; FRT82Bwts&lt;sup&gt;4&lt;/sup&gt;/S.T.</td>
<td>A15, RT grow better Membrane Mats (L. Ho)</td>
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<td>w; UAS-myrdmatsGFP; Sb/S.T.</td>
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<tr>
<td>w; UAS-matsGFP(III)</td>
<td>#15-2 (T. Shimuzi)</td>
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<td>w; AB1-Gal4; UAS-matsGFP(15-2)(III)/S.T.</td>
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<td>w; ey-Gal4; UAS-wts(6R)/S.T.</td>
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<td><strong>Yki (S97A)</strong></td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>48C2</td>
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<tr>
<td><strong>UAS-YAP (S94A, S127A)</strong></td>
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<tr>
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<td>6A</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>8A</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
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<td>w; P[w+]/SM1; +/Sb or TM6</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>31A</td>
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<tr>
<td>w; +/Adv or SM1; P[W+]/TM6</td>
<td>33A</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
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<tr>
<td><strong>YAP (S127A, WW1&amp;WW2)</strong></td>
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</tr>
<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>4A2</td>
</tr>
<tr>
<td>w, P[w+]/P[w+]or Y;</td>
<td>8C3</td>
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<tr>
<td>w; Adv/SM1; P[w+]/TM6</td>
<td>16A</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>17A1</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>20A1</td>
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Yki (WW1)

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<tr>
<th>w; P[w+]/SM1; +/Sb or TM6</th>
<th>20A2</th>
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<tbody>
<tr>
<td>w; P[w+]/Sm1; Sb/ or Tm6</td>
<td>20B</td>
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Yki (WW2)

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<th>w; P[w+]/SM1; +/Sb or TM6</th>
<th>2A2</th>
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<tbody>
<tr>
<td>w; Adv/SM1; P[w+]/TM6</td>
<td>4A2</td>
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<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>6A1</td>
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<tr>
<td>w; P[w+]/SM1; Sb/or TM6</td>
<td>6B1</td>
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<tr>
<td>w; P[w+]/SM1; +/- Sb or TM6</td>
<td>10A1</td>
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<tr>
<td>w; P[w+]/SM1; +/- TM6</td>
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<td>w, P[w+]/P[w+] or Y</td>
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<tr>
<td>w; P[w+]/SM1; +/- Sb or TM6</td>
<td>19B2</td>
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<tr>
<td>w; Adv/SM1; P[w+]/TM6</td>
<td>19D</td>
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Yki (WW1&WW2)

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<thead>
<tr>
<th>w; +/- SM1; P[w+]/TM6</th>
<th>19B</th>
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<tbody>
<tr>
<td>w; P[W+]/SM1; Sb/or TM6</td>
<td>21A</td>
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<tr>
<td>w; +/-Adv or SM1; P[w+]/TM6</td>
<td>21C</td>
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<tr>
<td>w; P[W+]/SM1; Sb/TM6</td>
<td>23A</td>
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<tr>
<td>w; P[w+]/SM1; +/- Sb or TM6</td>
<td>37B1</td>
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<tr>
<td>w; P[w+]/SM1; Sb/or TM6</td>
<td>49B</td>
</tr>
<tr>
<td>w; P[w+]/SM1; +/- Sb or TM6</td>
<td>58A</td>
</tr>
<tr>
<td>Strain</td>
<td>Quantity</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
</tr>
<tr>
<td>w; P[w+]/SM1; +/TM6</td>
<td>58B</td>
</tr>
<tr>
<td>w; P[w+]/SM1; +/Sb or TM6</td>
<td>67A</td>
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**DsRed/GFP-tagged Yki**

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<th>Strain</th>
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<th>Origin</th>
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<tbody>
<tr>
<td>w; UAS-YkiV5DSRed/SM1; Sb/or TM6</td>
<td>(5)3</td>
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</tr>
<tr>
<td>w; UAS-YkiV5DSRed/SM1; Sb/TM6</td>
<td>11 strong line</td>
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</tr>
<tr>
<td>w; UAS-YkiV5DSRed/SM1; Sb/or TM6</td>
<td>(11)6</td>
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<tr>
<td>w; UAS-YkiV5DSRed/SM1; Sb/TM6</td>
<td>(26)1</td>
<td></td>
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<tr>
<td>w; UAS-YkiV5DSRed/SM1; Sb/TM6</td>
<td>(38)12</td>
<td></td>
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<tr>
<td>w; Adv/or SM1; UAS-YkiV5DSRed/TM6</td>
<td>(93)4</td>
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<tr>
<td>w; Adv/SM1; UAS-YkiV5GFP/TM6</td>
<td>(17)2 homozygous lethal</td>
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<tr>
<td>w; Adv/SM1; UAS-YkiV5GFP/TM6</td>
<td>(94B)12</td>
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<tr>
<td>w; UAS-YkiV5GFP/SM1; Sb/TM6</td>
<td>(100)8</td>
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<tr>
<td>w; +; C5&gt;YkiV5GFP/S.T. (III)</td>
<td>(90)4</td>
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<td>w; UASYkiV5GFP; C5-Gal4/S.T.</td>
<td>(94A)10</td>
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<tr>
<td>w; GMR-Gal4; UAS-YkiV5GFP/S.T.</td>
<td>(90)4</td>
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<tr>
<td>w; GMR-Gal4; UASYkiV5DsRed/S.T. (III)</td>
<td>weak line</td>
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**Other Yki, YAP mutant lines**

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<td>D. Pan</td>
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<tr>
<td>w; UAS-YAP(W1W2)(4.35)/SM1; UAS-YAP(W1W2)(4.35)/TM6</td>
<td>X. Wei</td>
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<tr>
<td>w; UAS-YAP(II)(44.1)/S.T.</td>
<td>X. Wei</td>
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<tr>
<td>w; UAS-YkiV5(14.1)/SM1; Sb/TM6</td>
<td>X. Wei</td>
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<tr>
<td>w; UAS-YkiV5(III)(55.22) /S.T.</td>
<td>X. Wei</td>
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<tr>
<td>w; SM1/+; UAS-YkiV5(55.22)/TM6</td>
<td>X. Wei</td>
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</tr>
<tr>
<td>w; UAS-YkiV5(8.1); Sb/TM6</td>
<td>X. Wei</td>
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<tr>
<td>w; GMR-Gal4,UAS-YAP(61.1)(II)/S.T.</td>
<td>Weak</td>
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<tr>
<td>w; GMR-Gal4,UAS-Yki-V5(II)(8.1)/S.T.</td>
<td>(lost)</td>
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<tr>
<td>w; GMR-Gal4; UAS-Yki-V5(55.22) (III)/S.T.</td>
<td>X. Wei</td>
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</tr>
<tr>
<td>Genotype</td>
<td>Source</td>
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<td>-------------------------------------------------------------------------</td>
<td>-------------------------</td>
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<td>w; GMR-Gal4, UAS-Yki(17.4)(II)/S.T.</td>
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<tr>
<td>w; UAS-YAP(44.1)(I)/SM1</td>
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<tr>
<td>w; UAS-YAP(S127A)(8.2); Sb/S.T</td>
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<td>w; UAS-YAP(S127A)(36.1); +</td>
<td>X. Wei</td>
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<tr>
<td>w; UAS-YAP(S127A)(8.2); Sb/TM6</td>
<td>X. Wei</td>
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<tr>
<td>w; UAS-YAP(S127A)(36.1)/SM1; Sb/TM6</td>
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<tr>
<td>w; YAP(S94A)(31.2)/SM1; +/-</td>
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<tr>
<td>w; FRT42yki&lt;sup&gt;85&lt;/sup&gt;/Cyo</td>
<td>D. Pan</td>
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<tr>
<td>w; UAS-Yki(17.4)/SM1; Sb/TM6</td>
<td>X. Wei</td>
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<tr>
<td>w; UAS-Yki(15.3)/SM1; Sb/TM6</td>
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<td>X. Wei</td>
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<tr>
<td>w; UAS-YkiV5(11.2)/SM1; Sb/TM6</td>
<td>X. Wei</td>
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<tr>
<td><strong>sd, bantam lines</strong></td>
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<tr>
<td>yw;sd&lt;sup&gt;12&lt;/sup&gt;FRT18A/FM7a</td>
<td>#9371</td>
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<tr>
<td>w; +/-SM1; UAS-Sd/TM6</td>
<td>#9373</td>
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<tr>
<td>w; UAS-Sd/SM1; +/-TM6</td>
<td>#9374</td>
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<tr>
<td>w; GMR-Gal4; UAS-Sd(9373)/S.T.</td>
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<tr>
<td>w; GMR-Gal4; UAS-Sd(9374)/S.T.</td>
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<tr>
<td>w; UASYkiV5(8.1)/SM1; UAS-Sd(9373)/TM6</td>
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<tr>
<td>w; bantami/TM6B hu<code>, tb</code></td>
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<td>EPo3622 bantam</td>
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<td>yw; ban<code>/TM3, Ser</code></td>
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<td>Bantam UAS-C/TM6(L)</td>
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<td><strong>RNAi lines</strong></td>
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<tr>
<td>w; UAS–YkiRNAi/SM1; Sb/TM6</td>
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<td>w; UAS-YkiRNAi; FRT82B90E/S.T.</td>
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<td>hpo–RNAi (II)</td>
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<tr>
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<td>UAS-PTEN-RNAi(II)</td>
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<td>w; en-Gal4; UAS-Dicer/S.T.</td>
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**lacZ recombinant lines**

- w; UAS-Yki(8.1); 16.4-lacZ/S.T.  
- w; UAS-YAP(S127A)(36.1); 16.4-lacZ/S.T.  
- w; UAS-Yki(WW1&WW2) (8A); 16.4 -lacZ/S.T.  
- w; UAS-Yki(8.1); th^5c8/S.T.

**Akt signaling pathway lines**

**tGPH lines**

- w; tGPH(II); Sb/TM3 Ser**  
  #8163

- w; tGPH (III)  
  #8164

- w; Adv/SM1; tGPH/TM6
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<tr>
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<tr>
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**Akt pathway components lines**

| yw; UAS-Pi3k.Exel (II) (dp110) | #8266 WT |
| yw; UAS-Pi3k.Exel (III) (dp110) | #8287 WT |
| UAS-Pi3k.CAAX,y,w (dp110) | #8294 constitutively active |
| yw; UAS-InR.Exel (II) | #8262 WT fly InR |
| yw; UAS-InR.R418P (II) | #8250 constitutively active fly InR |
| yw; GMR-Akt1( II) | #8196 |
| GMR–Akt1.y,w (X) | #8195 |
| UAS–Akt1.y,w (X) | #8192 |
| yw; UAS–Akt1(II) | #8191 |
| ry,Akt<sup>1</sup>04226/TM3, ry` Sb Ser` | #11627 |
| yw; Akt<sup>1</sup>EY10012/TM3, Sb`Ser` | #19894 |

**Epistasis analysis of Akt and Hippo pathway components**

| w; UAS-Akt1RNAi; FRT82B90E/S.T. |  |
| w; UAS-Akt1RNAi; FRT82Bmats<sup>235</sup>/S.T. |  |
| w; UAS-dPi3kRNAi; FRT82B90E/S.T. |  |
| w; UAS-dPi3kRNAi; FRT82Bmats<sup>235</sup>/S.T. |  |

**Other Signaling Pathway Components**

<p>| w; UAS-ptC.J (III) | #5817 |
| w; UAS-Ras85D.K (II) | #5788 |
| w; UAS-Ras85D.V12 (III) | #4847 |
| yw; UAS-Egfr.B (III) | #5368 |
| yw; UAS-Egfr (DN); UAS-Egfr (DN) | #5364 |</p>
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<tr>
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Both TEAD-Binding and WW Domains Are Required for the Growth Stimulation and Oncogenic Transformation Activity of Yes-Associated Protein

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Abstract

The Yes-associated protein (YAP) transcription coactivator is a candidate human oncogene and a key regulator of organ size. It is phosphorylated and inhibited by the Hippo tumor suppressor pathway. TEAD family transcription factors were recently shown to play a key role in mediating the biological functions of YAP. Here, we show that the WW domain of YAP has a critical role in inducing a subset of YAP target genes independent of or in cooperation with TEAD. Mutation of the WW domains diminishes the ability of YAP to stimulate cell proliferation and oncogenic transformation. Inhibition of YAP oncogenic-transforming activity depends on intact serine residues 127 and 381, two sites that could be phosphorylated by the Hippo pathway. Furthermore, genetic experiments in Drosophila support that WW domains of YAP and Yki, the fly YAP homologue, have an important role in stimulating tissue growth. Our data suggest a model in which YAP induces gene expression and exerts its biological functions by interacting with transcription factors through both the TEAD-binding and WW domains. [Cancer Res 2009;69(3):1089–98]

Introduction

Yes-associated protein (YAP) is a transcription coactivator and a candidate human oncogene regulated by the Hippo pathway, a novel tumor suppressor pathway first characterized by Drosophila genetic studies (1–10). The Hippo pathway limits organ size in Drosophila by inhibiting Yki, the YAP homologue (11). Biochemical studies showed that Yki is directly phosphorylated and inhibited by the Wts protein kinase, which is phosphorylated and activated by the Hippo protein kinase (12, 13). Components of the Hippo pathway are highly conserved in mammals. Recent studies from our group and others have shown that YAP is phosphorylated and inhibited by the Lats tumor suppressor kinase, which is the mammalian homologue of Wts (14–16). Lats phosphorylates YAP on serine residue 127 in the HHRXXS motif, which results in 14-3-3 binding and cytoplasmic retention of YAP, therefore leading to YAP inhibition (14). This mechanism of YAP regulation is implicated in cell contact inhibition and tissue growth control (14, 17).

YAP is a potent growth promoter. Overexpression of YAP increases organ size in Drosophila and saturation cell density in NIH-3T3 cell culture (14). However, yap was termed a candidate oncogene only after it was shown to be in human chromosome 11q22 amplicon that is evident in several human cancers (18–21). Besides the genomic amplification, YAP expression and nuclear localization were also shown to be elevated in multiple types of human cancers (12, 14, 20, 22). Several experiments further confirmed that YAP has oncogenic function: YAP overexpression in MCF10A cells induces epithelial-mesenchymal transition (EMT), which is often associated with cancer metastasis (21); YAP cooperates with myc oncogene to stimulate tumor growth in nude mice (20); and more interestingly, transgenic mice with liver-specific YAP overexpression show a dramatic increase in liver size and eventually develop tumors (12, 23). The above evidence strongly indicates the function of YAP as an oncogene, although the mechanism by which YAP promotes oncogenesis is a question that remains to be answered.

YAP is a transcription coactivator, which itself has no DNA-binding activity. Recent studies from Drosophila and mammalian cells have shown that TEAD plays a critical role in mediating YAP-dependent gene induction and growth control (24–28). YAP and TEAD bind to a common set of promoters in MCF10A cells (27). Disruption of YAP-TEAD interaction or knockdown of TEAD attenuates the expression of many YAP target genes and blocks YAP-induced growth promotion and EMT (27). The Drosophila TEAD homologue, Scalloped (Sd), also interacts with Yki and is required for Yki to stimulate tissue growth (24–26). Collectively, TEAD is a key downstream transcription factor mediating YAP cellular function. However, in Drosophila, yki mutant cells have more severe growth defects than sd mutant cells (11, 29, 30), and overexpression of the Sd-binding–defective Yki-S97A elicits a reduced but still obvious overgrowth in Drosophila eyes and wings (27). Consistently, the TEAD-binding–defective YAP-S94A mutant can still induce expression of a fraction of YAP-regulated genes (27). These observations indicate that besides TEAD, additional transcription factors may be used by YAP/Yki to stimulate cell and tissue growth.

YAP has an NH2-terminal TEAD-binding domain (TBD) and a COOH-terminal transactivation domain, with one or two WW domains (two splicing variants, YAP1 and YAP2, respectively) in between (31). The WW domain is a protein-protein interaction module with two signature tryptophan (W) residues spaced 20 to 22 amino acids apart (32). It binds to ligands containing proline-rich sequences. For example, the PPXY motif represents the largest class of WW domain ligands. Interestingly, PPXY motif is present in a wide range of transcription factors, among which ErbB4 intracellular domain (33), RUNX2 (34), and p73 (35, 36) have already been reported to bind to YAP WW domain. However, it is
not clear if the WW domain, therefore any of the PPXY motif-containing transcription factors, mediates the gene induction and biological functions of YAP. The Lats kinase, which regulates YAP activity by direct phosphorylation, also contains one or two PPXY motifs (Lats2 has one and Lats1 has two PPXY). Therefore, the WW domain of YAP was also suggested to contribute to YAP inhibition by mediating interaction with Lats (15, 37).

In this report, we show that the WW domain of YAP is not essential for its inhibition by Lats. However, it is critical for induction of a subset of YAP target genes in cooperation with or independent of TEAD. Mutation of the WW domains diminishes the ability of YAP to promote cell proliferation, serum-independent growth, and oncogenic transformation. Interestingly, the WW domain is not essential for YAP to induce EMT in MCF10A cells, whereas TBD is required for both cell proliferation and EMT. The phosphorylation-defective YAP-5SA mutant is capable of transforming NIH-3T3 cells, and its oncogenic activity is inhibited by restoring either one of serine residues 127 or 381. Moreover, genetic experiments in Drosophila show a critical role of WW domains of YAP and Yki in stimulating tissue growth in vivo. Our study suggests that transcription factors interacting with the WW domains of YAP play an important role in mediating the oncogenic and growth promotion function of YAP.

Materials and Methods

Cell culture, transfection, and retroviral infection. HEK293 cells, HEK293-T cells, and NIH-3T3 cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen) and 50 μg/mL penicillin/streptomycin. MCF10A cells were cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20 ng/mL epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 100 ng/mL cholera toxin, and 50 μg/mL penicillin/streptomycin. Transfection with Lipofectamine was performed according to the manufacturer’s instructions.

To generate stable cells expressing wild-type (WT) or the indicated mutant YAP proteins, retrovirus infection was performed by transfecting 293 Phoenix retrovirus packaging cells with empty vector or pQCXIH-YAP constructs. Forty-eight hours after transfection, retroviral supernatant was supplemented with 5 μg/mL polybrene, filtered through a 0.45-μm filter, and used to infect MCF10A or NIH-3T3 cells. Thirty-six hours after infection, cells were selected with 200 μg/mL hygromycin (Roche) in culture medium.

Three-dimensional culture of MCF10A cells. The three-dimensional culture of MCF10A cells was done as described elsewhere (38). Briefly, growth factor–reduced Matrigel was layered onto eight-well glass chamber slide to make a reconstituted basement membrane. MCF10A cells were seeded on top of that at a concentration of 5,000 per well in assay medium containing 2% Matrigel and 5 ng/mL EGF. Cells were cultured in a 5% CO2 humidified incubator at 37°C. The medium was replaced every 4 d.

Figure 1. WW domains of YAP mediate gene induction but are not required for YAP inhibition by Lats. A, WW domains of YAP are not required for the inhibition by Lats2. Indicated plasmids were cotransfected with a 5× UAS-luciferase reporter, Gal4-TEAD4, and a CMV-β-gal construct into 293T cells. Luciferase activity was measured and normalized to β-galactosidase activity. W1W2 denotes mutation of the two WW domains in YAP; Lats2-YA denotes the Lats2 PPXY motif mutant. B, the TBD and WW domains mediate the activation of different transcription factors by YAP. YAP WT or mutants were cotransfected with the indicated transcription factors into 293T cells. ErbB4 intracellular domain and TEAD4 were Gal4 fused and were cotransfected with a 5× UAS-luciferase reporter. RUNX2 was cotransfected with the 6× OSE2-luciferase reporter. Luciferase activity was measured and normalized to activity of cotransfected β-galactosidase. C, both the TBD and WW domains are involved in YAP-induced gene expression. MCF10A cells stably expressing YAP WT or mutants were generated by retroviral infection. The expression of indicated genes was determined by quantitative reverse transcription-PCR and compared with vector control cells.
Luciferase assay. For the luciferase reporter assay, HEK293-T cells were seeded in 12-well plates. Luciferase reporter, cytomegalovirus (CMV)-\(\beta\)-gal, and indicated plasmids were cotransfected. Thirty-six hours after transfection, cells were lysed and luciferase activity was assayed using the enhanced luciferase assay kit obtained from BD Biosciences following the manufacturer's instructions. All luciferase activities were normalized to \(\beta\)-galactosidase activity.

RNA isolation and real-time PCR. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen). cDNA was synthesized by reverse transcription using random hexamers and subjected to real-time PCR with gene-specific primers in the presence of SYBR Green (Applied Biosystems). Relative abundance of mRNA was calculated by normalization to \(\beta\)-galactosidase activity.

Colonies formation assay. Colony formation assay was performed as briefly described below. NIH-3T3 fibroblasts were seeded on six-well plates at a density of \(10^5\) cells per well and then transfected with YAP WT or mutants using Fugene6 (Roche) according to the manufacturer's instructions. After 2 d, cells were replated onto 10-cm dish and maintained in DMEM supplemented with 5% FBS for 2 to 3 wk until foci were evident. Cells were fixed with 10% acetic acid and 10% methanol, and then colonies were stained with 1% crystal violet and counted.

In vitro kinase assay. For Lats2 kinase assays, HEK293 cells were cotransfected with HA-Lats2 and Flag-Mst2 to express active Lats protein. Forty-eight hours after transfection, cells were lysed with lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 10 mmol/L pyrophosphate, 10 mmol/L glycerophosphate, 50 mmol/L NaF, 1.5 mmol/L Na<sub>3</sub>VO<sub>4</sub>, protease inhibitor cocktail (Roche), 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride] and immunoprecipitated with anti-HA antibodies. The immunoprecipitates were washed thrice with lysis buffer, once with buffer containing 40 mmol/L HEPES and 200 mmol/L NaCl, and once with kinase assay buffer (50 mmol/L HEPES, 50 mmol/L potassium acetate, 5 mmol/L MgCl<sub>2</sub>). The immunoprecipitated Lats2 was subjected to a kinase assay in the presence of 500 \(\mu\)mol/L cold ATP, 10 \(\mu\)Ci \([\gamma-32P]\)ATP, and 1 \(\mu\)g of bacterially expressed WT or mutant glutathione.

Figure 2. The WW domain is required for YAP-induced overgrowth but not EMT. A, YAP-W1W2 is defective in promoting cell growth. Growth curve of NIH-3T3 stable cells with expression of vector, YAP, and YAP-W1W2 was determined. Top, the expression of YAP WT or W1W2 mutant was shown by Western blot. B, WW domain mutant of YAP is comprised in inducing enlarged acini of MCF10A cells in three-dimensional culture. Indicated MCF10A stable cells were cultured in 3D on reconstituted basement membrane for 16 d before pictures were taken. Top, the ectopic expression of YAP was shown by Western blot. C, WW domains of YAP are not required for inducing an EMT-like morphology in MCF10A cells. The morphology of indicated MCF10A stable cells in tissue culture was recorded to show their difference. D, the TBD but not WW domain is required for reducing membrane E-cadherin and cortical actin. Indicated MCF10A stable cells were stained by anti-E-cadherin (green), rhodamine-phalloidin (red), and 4',6-diamidino-2-phenylindole (DAPI; blue).
S-transferase (GST)-YAP proteins as substrate at 30°C for 30 min with a gentle shaking. The reaction was terminated by adding SDS sample buffer and subjected to SDS-PAGE and autoradiography.

**Drosophila genetics.** For in vivo functional analysis of YAP/Yki, full-length cDNAs of YAP or yki were cloned into a transformation vector pUAST (39). Multiple transgenic fly lines were generated for each of the following DNA constructs: pUAS-Flag-YAP<sup>S127A/W1W2</sup> (15 lines) and pUAS-yki<sup>W1W2-V5</sup> (31 lines), pUAS-Flag-YAP<sup>S127A</sup> and pUAS-yki<sup>V5</sup> were previously reported (14). C5-Gal4 and GMR-Gal4 drive wing- and eye-specific expression of UAS transgenes, respectively. For adult wing size analysis, at least 30 wings of each genotype were used for analysis. For clonal overexpression analysis of Yki and YAP, corresponding UAS transgenic flies were crossed with w, hsFLP; act>y+>Gal4; UAS-GFP/TM6B and progenies were raised at 20°C. Four days later, the flies were heat treated at 31°C for 1 h and then left at 20°C for another 3 d. Late third instar larvae were dissected and wing imaginal discs were fixed in 8% paraformaldehyde-lysine-phosphate buffer for 45 min at 4°C. Green fluorescent protein (GFP) signal was observed by confocal microscopy. Immunofluorescent staining of mid-pupal eye discs was done with mouse anti-Discs large (Dlg; 1:300; Developmental Studies Hybridoma Bank) as primary antibody and Alexa Fluor 488 (1:300; Molecular Probes) as secondary antibody. Scanning electron microscopy was done to reveal adult retinal phenotypes.

**Results**

**WW domains are not required for YAP inhibition by Lats.** It has been suggested that the WW domains of YAP may bind to the PPXY motifs of Lats, therefore playing a role in recruiting Lats to YAP (15, 37). To test this possibility, we examined the effect of Lats on YAP WW domain mutant in reporter assay. Our data show that with Mob cotransfection, Lats could potently inhibit both WT and WW domain mutant YAP (Fig. 1A), indicating that the WW domains of YAP are not required for its inhibition by Lats. Similar results were obtained without Mob cotransfection, although the inhibition on both YAP-WT and W1W2 is less potent (data not shown). Consistently, mutation of the PPXY motif in Lats2 did not abolish its ability to inhibit YAP (Fig. 1A). These results argue against a model in which the WW domain mediates the inhibition of YAP by Lats.

**Both the TBD and WW domains of YAP are involved in gene induction.** It is possible that WW domains of YAP mediate interactions with transcription factors, therefore regulating gene expression. Several transcription factors, such as ErbB4 and

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**Figure 3.** Both the TBD and WW domains are required for YAP-induced serum-independent growth of fibroblasts. Stable pools of NIH-3T3 fibroblasts expressing vector control (Vec) and the indicated YAP mutant proteins [WT, TEAD binding defective (S94A), WW domains mutant (W1W2)] were grown in medium containing low (0.5%) or normal (10%) serum. Cells were seeded at the same density (2.5 x 10<sup>4</sup>) and then their morphology (A) as well as growth rate (B) were monitored.
RUNX2, have been reported to be activated by YAP (33, 34). We examined the involvement of different domains of YAP in activation of these transcription factors. We previously identified serine 94 of YAP as an essential residue for its interaction with TEADs (27). As expected, S94A mutation of YAP completely abolished its ability to activate TEAD4 (Fig. 1B). Interestingly, YAP S94Amutant is capable of fully activating both ErbB4 and RUNX2, indicating that the TBD of YAP is not involved in its interaction with either ErbB4 or RUNX2. YAP has two WW domains, the first one of which has been implicated in interaction with ErbB4 and RUNX2, indicating that the TBD of YAP is not involved in its interaction with either ErbB4 or RUNX2. YAP has two WW domains, the first one of which has been implicated in interaction with ErbB4 and RUNX2, indicating that the TBD of YAP is not involved in its interaction with either ErbB4 or RUNX2 (33, 34). We found that mutation of the first (W1) or both (W1W2) WW domains in YAP abolished its ability to activate ErbB4 or RUNX2, whereas mutation of the second WW domain (W2) only modestly decreased this activity (Fig. 1B). However, mutation of the WW domains does not attenuate the activity of YAP on TEAD4. These data indicate that YAP uses two distinct domains, the TBD and WW, to activate different downstream target transcription factors.

As we previously reported, the TBD of YAP is required for induction of many YAP-inducible genes in MCF10A cells (27). Here, we compared gene expression profiles of MCF10A cells overexpressing YAP WT or WW domain mutant. Interestingly, a subset of YAP-inducible genes requires the intact WW domains in YAP (Supplementary Table S1). The expression of some of those genes was confirmed by real-time PCR as shown in Fig. 1C. Induction of ALPP largely depends on the WW domains but not the TBD. In contrast, induction of CTGF is absolutely dependent on TBD but not WW domains. Moreover, induction of ITGB2 and PIK3C2B requires both the TBD and WW domains (Fig. 1C). Therefore, it is clear that WW domains are essential for the expression of a subset of YAP-inducible genes, some of which also depend on the TBD.

The WW domain is required for YAP-induced proliferation but not EMT. YAP expression stimulates cell growth in both NIH-3T3 fibroblast and MCF10A (21, 27), a human mammary epithelial cell line. We tested the function of YAP WW domains in stimulating cell growth. Stable expression of WT YAP significantly increased NIH-3T3 cell growth compared with the vector control cells (Fig. 2A). However, expression of YAP-W1W2 mutant failed to do so. The effect of YAP expression on MCF10A cell growth was assayed in three-dimensional culture on reconstituted basement membrane. Expression of YAP-5SA, an active mutant with elimination of all five HXRXXS phosphorylation sites, strongly increased the acini size in three-dimensional culture (Fig. 2B). In

Figure 4. Phosphorylation of serine 127 or 381 is sufficient to inhibit transformation potential of YAP. A, YAP-5SA elicits a transformed morphology in NIH-3T3 cells. NIH-3T3 fibroblasts expressing vector (Vec), YAP-WT, or 5SA were seeded at the same density in medium containing 0.5% serum and their morphology after 4 d was shown. B, serine 127 and 381 were sufficient to confer inhibition of YAP-induced colony formation. Colony formation assays were performed using vector control or indicated YAP constructs. Colonies were visualized with crystal violet staining and pictured. The absence of colonies in the plate of YAP-4SA/S61 transfected cells is likely due to lack of YAP-4SA/S61 expression (data not shown). C, quantification of the colony number shown in B. Colony number in assay using YAP-S127A is also shown. D, all five HXRXXS motifs of YAP could be phosphorylated by Lats in vitro. WT YAP and various phosphorylation mutants were purified from bacteria as GST fusion proteins and were subjected to kinase assays in the presence of [32P]ATP with immunoprecipitated Lats from HEK293 cells. Phosphorylation of YAP was detected by [32P] incorporation (top) and GST-YAP input was shown by Coomassie blue staining (middle). Bottom, the relative [32P] incorporation was quantified.
contrast, mutation of WW domains significantly attenuated this activity of YAP-5SA.

Previously, it had been reported that YAP expression promotes EMT in MCF10A cells (27). We compared the cell morphology of MCF10A cells stably expressing YAP-5SA or YAP-5SA-W1W2. Surprisingly, cells expressing YAP-5SA-W1W2 display EMT-like morphologic changes similar to those induced by YAP-5SA (Fig. 2C). In contrast, mutation of S94 or deletion of the COOH-terminal activation domain abolished this activity of YAP-5SA. YAP-induced EMT in MCF10A cells was also shown by the loss of cell-cell junction localized E-cadherin and the switch from cortical actin to stress fibers (Fig. 2D). These alterations were induced by WT YAP as well as YAP-W1W2 but not YAP-S94A mutant (Fig. 2D).

Our results suggest that the WW domain is not required for YAP to induce EMT but is important for YAP to promote proliferation in MCF10A cells.

Both the TBD and WW domains are required for cell growth in low serum medium. YAP is a candidate oncogene capable of promoting tumor formation, which requires the cell to not only proliferate faster but also gain other characters, such as self-sufficiency of growth signals, a hallmark of cancer (40). We tested the ability of YAP WT or mutants to induce NIH-3T3 cell serum-independent growth. In medium containing 0.5% serum, NIH-3T3 cells with vector control cannot proliferate. However, expression of WT or active forms of YAP confers NIH-3T3 cells proliferation potential in low serum medium (Fig. 3A and B). This is consistent with the oncogenic function of YAP. In contrast, the TBD-defective or WW domain–defective mutants completely lost the ability to promote serum-independent growth. In fact, under low serum conditions, the YAP-S94A–expressing or W1W2-expressing cells displayed a significant decrease in cell numbers, likely due to apoptosis, whereas the vector control cells remain viable (Fig. 3A and B). However, under normal culture conditions (10% serum), neither YAP-S94A nor YAP-W1W2 expression induced cell death. These results show that both the TBD and WW domains are essential for YAP to promote self-sufficiency of growth signals in NIH-3T3 cells.

The transformation potential of YAP is inhibited by phosphorylation of serine 127 or 381. YAP is a candidate human oncogene amplified in multiple cancers or cancer cell lines (18–21). Elevated YAP expression and nuclear localization is also observed in human cancers (Supplementary Fig. S1A). To further establish the function of WW domains in the oncogenic potential of YAP, we first tested if YAP could transform NIH-3T3 cells. Surprisingly, expression of WT YAP does not induce a transforming morphology (Fig. 4A). We have previously shown that Lats phosphorylates YAP to inhibit its transactivation and growth promotion activity (14). It is possible that YAP oncogenic potential is also inhibited by Lats-dependent phosphorylation. Mutation of all five serine residues (61, 109, 127, 164, and 381) matching Lats phosphorylation target consensus (HXRXXS) to alanines (YAP-5SA) was reported to make YAP resistant to inhibition by Lats (14). Interestingly, YAP-5SA not only is more potent in stimulating cell proliferation but also causes transformation properties in NIH-3T3 cells (Figs. 3B and 4A), such as growing on top of each other, indicating the loss of contact inhibition.

We further performed colony formation assays, which are well established to examine oncogenic potential. As expected, YAP-5SA could potently induce colony formation, whereas YAP WT could not (Fig. 4B and C), which indicates that the oncogenic activity of YAP is inhibited by phosphorylation on at least some of the five sites. However, it is not clear which ones of the five possible sites are critical. To answer this question, we restored individual serine in the YAP-5SA mutant, resulting in YAP-4SA proteins retaining a single putative phosphorylation site. Restoration of serine 127 (4SA/S127) and 381 (4SA/S381) abolished the oncogenic potential.
of YAP-5SA. In contrast, restoration of serine residues 109 (4SA/S109) and 164 (4SA/S164) did not abolish the transforming activity of YAP-5SA/S61. Although YAP-4SA/S61 transfected cells did not form any colony, we could not conclude the importance of S61 due to lack of expression from this DNA construct. These data suggest that phosphorylation of serine 127 or 381 is sufficient to inhibit YAP, therefore abolishing its transformation activity. Consistently, although phosphorylation of serine 127 is known to mediate YAP...

Figure 6. The WW domain plays a critical role in YAP/Yki-induced tissue growth. A, the TBD and WW domain mutants of YAP/Yki are compromised in promoting wing tissue growth. Overexpression of various yki and yap transgenes was driven by C5-Gal4. Genotypes of the fly tissues are indicated. f, arrows, two gaps along the fourth longitudinal vein. B, WW domain mutants of Yki and YAP are compromised in inducing clone expansion. Wing imaginal discs containing 72-h-old control (a) or various YAP/Yki-overexpressing clones (b–e) were generated by flip-out and positively marked by GFP. Genotypes of the fly tissues are hsFLP+/act>y->Gal4, UAS-GFP127A (a), hsFLP+/act>y->Gal4, UAS-GFP127A/UAS-yki-V5 (b), hsFLP+/act>y->Gal4, UAS-GFP127A/UAS-ykiT127A-V5 (c), hsFLP+/act>y->Gal4, UAS-GFP127A/UAS-Flag-YAP127A (d), and hsFLP+/act>y->Gal4, UAS-GFP127A/UAS-Flag-YAP127A/W1W2 (e). C, the WW domains are important for Yki- and YAP-induced increase of eye size and disruption of retinal patterning. Genotypes of the fly tissues are WT (Canton S; a), GMR-Gal4/UAS-yki-V5 (b), GMR-Gal4/UAS-ykiW1W2-V5 (c), GMR-Gal4/UAS-Flag-YAPS127A (d), and GMR-Gal4/UAS-Flag-YAPS127A/W1W2 (e). D, proposed model of YAP/Yki transcription factor interaction under negative regulation by the Hippo pathway. YAP/Yki interacts with TEAD family transcription factors through the TBD, and with PPXY motif-containing transcription factors through the WW domains. By these two folds, YAP/Yki activates gene expression, and therefore stimulates growth and promotes oncogenic transformation. YBD, YAP-binding domain; DBD, DNA-binding domain; AD, activation domain. For the Hippo pathway components, their names in both mammals and Drosophila are given if different. Dashed arrows, unknown biochemical mechanisms.
inhibition, YAP-S127A single site mutant is not able to transform NIH-3T3 cells (Fig. 4C).

S127 of YAP is directly phosphorylated by Lats (14). We performed in vitro kinase assay to test if S381 is also a direct Lats target site. Lats could potently phosphorylate WT YAP but has little activity toward YAP-S5A (Fig. 4D). All YAP-5SA mutants could be phosphorylated by Lats with varying efficiency. These data suggest that all five Lats target consensus phosphorylation sites could be phosphorylated by Lats at least in vitro.

Using the available phosphorylated YAP S127 antibody, we compared YAP phosphorylation in several cell lines. Among them, MCF10A, a noncancerous cell line, showed the highest phosphorylation level, and ACHN, a cancer cell line showing loss of contact inhibition, has very little YAP phosphorylation (Supplementary Fig. S1B). The impaired YAP phosphorylation in ACHN is likely due to mutation of Sav, a key component of the Hippo pathway (41). Collectively, YAP is capable of transforming NIH-3T3 cells, which is inhibited by phosphorylation on the Hippo pathway target sites, and dysregulation of YAP phosphorylation is observed in cancer cells.

Both the TBD and WW domains are important for the oncogenic activity of YAP. How YAP activates gene expression to promote oncogenesis is not clear. Based on the ability of YAP-S5A to transform NIH-3T3 cells, we tested the role of the TBD and WW domains, two domains mediating YAP-transcription factor interactions, in YAP-induced oncogenic transformation. Either the TBD or the WW domain was mutated in YAP-5SA, and their transformation activity was examined. As expected, WT, S94A, and W1W2 mutant YAP could not transform NIH-3T3 cells (Fig. 5A and B). However, in the YAP-5SA background, mutation of either the TBD or WW domains significantly decreased the number of colonies induced, indicating the importance of both domains in the oncogenic transformation activity of YAP.

TEAD/Sd-binding and WW domains are important for YAP/Yki to promote tissue growth in Drosophila. To examine the significance of the TBD and WW domains in YAP-induced tissue growth, we generated transgenic flies that express human YAP, YAP-S94A, YAP-W1W2, YAP-S127A, YAP-S127A/S94A, or YAP-S127A/W1W2 in developing wings. Similar constructs derived from fly Yki were also used for in vivo functional analysis. Expression of human YAP during Drosophila wing development increased the wing size by 14% (Fig. 6A, a and f; S2A). Morphology of 4% of the YAP-expressing wings was severely disrupted, and therefore, such flies were not included for wing size analysis. However, overexpression of YAP-S94A or YAP-W1W2 did not show significant change of wing size compared with the control flies (Fig. 6A, g and h; Supplementary Fig. S2A). In addition to the increase of wing size, YAP caused patterning defect of the wings, with the fourth longitudinal vein broken into three segments (Fig. 6A, f). This phenotype was not observed in YAP-S94A or YAP-W1W2 flies (Fig. 6A, g and h). As expected, active YAP-S127A was highly potent to cause severe malformation of the wing with large air bubbles in between apical and basal layers, which made it impossible to correctly measure the wing size (Fig. 6A, i). Mutation of S94A or W1W2 dramatically decreased the activity of YAP-S127A, so that the size and morphology of their wings was similar to that of control flies (Fig. 6A, j and k; Supplementary Fig. S2A). In case of fly Yki, its overexpression significantly increased the wing size by 27% (Fig. 6A, b; Supplementary Fig. S2A) and ~80% of the wings were too malformed to be measured correctly (Fig. 6A, e). Both S97A and W1W2 mutations reduced Yki activity, as wings of Yki-S97A and Yki-W1W2 flies were only 19% and 7% larger than WT controls (Fig. 6A, c and d; Supplementary Fig. S2A). Thus, both TEAD/Sd-binding and WW domains are critical for YAP and Yki proteins to promote tissue growth and control organ size.

The functional significance of Yki and YAP WW domains was further investigated in two additional assays. First, Yki/YAP and their derivatives were clonally expressed and their ability to promote clone expansion in wing discs was monitored. Compared with WT controls, both Yki and YAP-S127A strongly stimulated clone expansion so that individual clones as well as the entire wing discs were larger (Fig. 6B, a, b, and d). However, mutations in WW domains greatly reduced the activity of Yki and YAP-S127A as both the average clone size and wing disc size are similar to those of WT controls (Fig. 6B, a–e). In the second assay, both Yki-W1W2 and YAP-S127A/W1W2 were much less potent in increasing the adult eye size and disrupting retinal patterning (Fig. 6C, a–e). As expected, they were also less potent than Yki and YAP-S127A, respectively, in increasing the number of interommatidial cells (Supplementary Fig. S2B, a–e). These results further support our hypothesis that WW domains are important for the growth-promoting activity of Yki and YAP.

Discussion

YAP is a candidate oncogene that also regulates organ size. However, the mechanism by which YAP regulates oncogenesis and organ size is not well understood. Recent studies have shown that the TEAD family transcription factors play a critical role in mediating YAP-dependent gene induction, growth promotion, and transformation (27). However, we also observed that a subset of YAP target genes could be induced by the TEAD-binding–defective YAP-S94A mutant (27). Furthermore, Drosophila genetics study also showed that yki mutant cells have more severe growth defects than sd mutant cells (11, 29, 30), and overexpression of the Sd-binding–defective Yki-S97A elicits a reduced but still obvious overgrowth in Drosophila eyes and wings (27). These observations suggest that there are other transcription factors mediating YAP-induced gene expression and biological functions. WW domains are the most obvious candidate to mediate interactions with other transcription factors. In this study, we established the functional importance of YAP/Yki WW domains in gene expression induction, growth promotion, and oncogenic transformation.

The WW domain of YAP has been suggested to interact with Lats (15, 37), which phosphorylates and inhibits YAP. However, our study suggests a positive role of YAP WW domains in stimulating cell proliferation and oncogenic transformation in vitro and to promote tissue overgrowth in vivo. We showed that WW domains are not required for YAP inhibition by Lats. Furthermore, the PPXY motif of Lats is also dispensable for YAP inhibition. Although recent articles have documented the importance of WW domain in YAP and PPXY motif in Lats for their interaction, the authors also noticed that YAP fragments without the WW domain could still be phosphorylated by Lats (15), which is consistent with our observation that the WW domain is not required for YAP inhibition by Lats.

We characterized the oncogenic activity of YAP. YAP expression is elevated in several human cancers as shown by human cancer tissue microarray staining. Expression of WT YAP enhances proliferation rate and confers serum-independent growth in NIH-3T3 cells. The phosphorylation-defective YAP-S5A, but not the WT YAP, potently transforms NIH-3T3 fibroblasts. These data support
YAP as an oncogene negatively regulated by phosphorylation. Furthermore, mutation of either the TBD or WW domains significantly attenuates the transformation potential of YAP and largely represses YAP/Yki-induced tissue overgrowth in Drosophila. Together, as shown in Fig. 6D, we propose that under negative regulation by the Hippo pathway, YAP/Yki interacts with TEAD and PPXY motif-containing transcription factors through the TBD and WW domains, respectively, to induce gene expression that leads to growth stimulation and oncogenic transformation. Several transcription factors, such as ErbB4 cytoplasmic domain, RUNX2, and p73, have been reported to interact with YAP through the WW domain (33–35), although their biological significance was not clear. p73, a p53 family protein, has growth-inhibitory and apoptotic functions, therefore is unlikely to mediate the growth-promoting and oncogenic function of YAP. Knockdown of ErbB4 does not affect proliferation of ACHN cells. All three RUNX family members have a conserved PPXY motif (34). Efforts to simultaneously knock down these three proteins were unsuccessful (data not shown). There are actually more PPXY motif-containing transcription factors in the human genome, which could be potential YAP targets. Future studies are in need to identify the critical target transcription factors that interact with the WW domain of YAP to mediate its function.

It is worth noting that YAP-S94A or YAP-W1W2 mutant not just fails to support serum-independent growth but rather promotes cell death in low serum condition. In contrast, neither of them induces cell death in medium supplemented with 10% serum. There are two possible explanations. First, expression of YAP-S94A or YAP-W1W2 imposes a dominant-negative effect on the expression of some YAP target genes important for serum-independent growth. Expression of such a gene is likely to require both the TBD and WW domains. For example, decreased expression of PIK3C2B was seen by expression of either YAP-S94A or W1W2 (Fig. 1C). Second, it is also possible that an imbalanced induction of the TBD-dependent or WW domain--dependent YAP target genes induces apoptosis in low serum condition.

Besides characterizing the YAP transcription factor interaction domains, this report further clarifies the importance of the five possible Lats phosphorylation sites on YAP in regulation of its transformation potential. Using YAP-4SA proteins retaining a single HXXXXS site, we found that YAP transformation potential is inhibited if serine 127 or 381 is intact. This result suggests that phosphorylation on either one of these residues is sufficient to inhibit the oncogenic activity of YAP, and decreased YAP phosphorylation is observed in ACHN cancer cell line. Phosphorylation of S127 by Lats creates a 14-3-3 binding site to induce YAP cytoplasmic translocation (14). However, the mechanism by which phosphorylation of S381 inhibits YAP requires further study.

The Hippo-YAP pathway is a new connection between control of organ size and cancer. Elucidation of the mechanism of YAP-induced gene expression, growth promotion, and oncogenic transformation is of immediate importance. In this study, we established the function of YAP WW domains in these processes, which might be a new target of pharmacologic intervention in treating human cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Evolution of the \textit{mob} Gene Family

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Abstract: Mob proteins from distantly related eukaryotic species share very high sequence similarity and they are characteristic of a conserved Mob domain with around 180 amino-acid residues in length. However, the evolutionary relationship of \textit{mob} family genes has not been extensively investigated. Through a phylogenetic approach, we have conducted a comprehensive evolutionary analysis of the \textit{mob} gene family. Here we show that over 270 \textit{mob} family members from protists to animals can be organized in four distinct groups. This classification is strongly supported by the analysis of \textit{mob} exon-intron structures. Moreover, the conservation and divergence patterns of different groups of Mob proteins have been elucidated. Structural information and the identification of fixed amino acid substitutions provide evidence about the putative significance of specific residues in the structural integrity and/or molecular functions of Mob proteins. Thus, this study reveals the evolutionary history of \textit{mob} gene family and provides a basis for functional studies of Mob proteins.

Keywords: Gene evolution, \textit{mob} domain, \textit{mob} gene family, \textit{mats} - \textit{mob} as tumor suppressor, hippo signaling.

INTRODUCTION

The first member of the \textit{mob} (Mps one binder) gene family, \textit{mob1}, was identified in budding yeast as a critical regulator of mitosis [1]. In fission yeast, \textit{mob1} is involved in regulating processes such as cytokinesis [2]. These yeast Mob1 proteins bind to and stimulate the activities of Dbf2 and Sid2 protein kinases [3-5]. The other known Mob protein in yeast, Mob2, can similarly associate with and activate the kinase activities of Dbf2-related protein (Cbk1) and Sid2-related protein (Orb6) [6-8]. From these early studies, we began to understand that Mob proteins are critical intra-cellular signaling molecules and can function to regulate catalytic activity of certain protein kinases.

In human cells, Mob1-related proteins have been identified and shown to positively regulate Dbf2 homologues, NDR (nuclear Dbf2-related) protein kinases [9-11]. Similarly, the Drosophila Mob family proteins have been found to function as binding partners of fly Ndr family protein kinases Warts (Wts)/Lats (Large tumor suppressor) and Tri-cornered (Trc) [12, 13]. Moreover, the Drosophila \textit{mob1/mob} as tumor suppressor (\textit{mats}) (CG13852) was discovered as a growth inhibitor critical for cell proliferation and apoptotic control, and a human \textit{mats} ortholog can functionally replace the fly \textit{mats} gene to regulate tissue growth [12]. Studies on plant species also demonstrated the presence of Mob1-like genes and their putative function in cytokinesis [14-16]. Their cell-cycle-regulated expression patterns and subcellular localizations indicate these Mob-like proteins function in cell proliferation and programmed cell death [16]. Therefore, Mob proteins are functionally important in both single cell and multi-cellular eukaryotes.

Mob proteins share high sequence similarity and they are characteristic of a conserved domain \textit{Mob1}_phocean (pfam 03637) with around 180 amino acid residues in length (for simplicity, here we will call it the Mob domain). Mob family proteins are usually small and contain no other known structural motifs. The structural analyses of human, \textit{Xenopus laevis} and yeast Mob1 proteins have revealed a central four-helix bundle stabilized by a zinc ion [17-19]. Moreover, they have identified an evolutionarily conserved acidic surface by which Mob proteins might interact with Ndr family protein kinases through electrostatic interactions.

Considering their high sequence similarity across distantly related species, we aimed to carry out a comprehensive evolutionary analysis to investigate the evolutionary relationship among all \textit{mob} genes. Toward this goal, we have identified more than 270 \textit{mob} family members from protists to animals, and conducted phylogenetic analysis. The study of the exon-intron structures elucidated the conservation and divergence patterns of different groups of \textit{mob} genes. Structural information and the identification of fixed amino acid substitutions provided evidence about the putative significance of certain structural features or specific residues in the structural integrity and/or molecular functions of Mob proteins. Thus, this study offers not only the knowledge about the evolutionary history of \textit{mob} gene family but also a basis for the prediction of biological functions of Mob proteins in eukaryotes.

MATERIALS AND METHODS

Extraction of Sequences

Mob protein sequences were obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) and used as queries for BLASTp and tBLASTn searches against NCBI databases (http://www.ncbi.nih.gov) and Ensembl databases (http://www.ensembl.org/index.html). Some Mob proteins were identified by searching genome sequences from organism-specific web-
sites (Dictybase - http://dictygenome.org, the Giardia lamblia Genome Database - www.mbl.edu/Giardia, and Ciona intestinalis Genome - http://genome.jgi-psf.org/Ciona). Only sequences that have more than 50 percent coverage of query lengths were kept. Truncated sequences and redundant sequences that have more than 50 percent coverage of query lengths were kept. Truncated sequences and redundant sequences were eliminated. The final 73 sequences used for this study are summarized in Supplementary Table S1 with information on species, gene names and accession numbers.

**Sequence Alignments**

Initially, full-length protein sequences were aligned using Multiple Alignment Mode in ClustalX1.83 with default parameter setting. We used MEGA3.1 to generate a preliminary neighbor-joining (NJ) tree for observing a classification pattern of Mob family. Both N-terminus and C-terminus were shown to have poor sequence conservation compared to the Mob domain region. This is the only domain found in Mob proteins and it comprises about 80 percent of the full protein length. We used Mob domain sequences for further phylogenetic analysis. We also did individual alignment for each subgroup and profile alignments (ClustalX) for other analyses, such as identification of conserved substitutions and conservation mapping.

**Phylogenetic Analysis**

Multiple alignment results of Mob domain sequences were subject to neighbor-joining and maximum parsimony phylogeny reconstruction using MEGA3.1 and PHYLIP. NJ tree was rooted with a Mob from Diplomonadida (Giardia lamblia) and constructed using p-distances with complete elimination of alignment gaps. One hundred forty-four amino acids of the Mob domain were used. The reliability of the resulting NJ and MP trees was tested by 1000 bootstrap resamplings.

**Exon-Intron Structures**

The exon-intron structures of mob genes from representative organisms were determined by mRNA-to-genomic alignment using Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/spideyweb.cgi) or directly obtained from Ensembl web pages.

**Expression Analysis of Human MOB Genes**

The expression data of H1 histone member 0 (H1_0) and all human MOB genes were obtained from EST profiles of UniGene in NCBI. Updated EST data was collected in August of 2008 (Table S2).

**RESULTS**

**mob Genes are Present Widely in Eukaryotes**

Since the discovery of the first mob family member in yeast [1], more than 270 mob genes have been found in a variety of eukaryotes from the primitive protists to plants and animals. Two mob genes, mob1 and mob2, exist in single cell eukaryotes such as budding yeast S. cerevisiae. Neurospora has one more mob gene in addition to the mob1-like and mob2-like genes. Protists such as Giardia have at least three mob genes, which is comparable to the mob gene number in invertebrates D. melanogaster (four) and C. elegans (four). Generally, vertebrates have more mob family members. For example, there are seven mob genes in the human, mouse, rat, zebrafish and fugu genomes, eight in frog and five in chicken genomes. Because mob genes are only found in eukaryotes, the mob gene family appears to be an innovation of eukaryotic organisms.

**Evolutionary Relationships of mob Family Genes**

Phylogenetic analysis identified four major groups of mob genes (Fig. 1). Protists such as Giardia and Entamoeba have mob genes in almost all groups, indicating that gene duplications giving rise to major mob family members occurred very early in evolution. Although each group has only one mob gene from invertebrates, Group I and Group II have at least two mob genes from most vertebrates. It suggests that additional gene duplications have resulted in the different mob genes repertoire of vertebrates.

Group I, also called Mats group [12], can be further divided into two subgroups, Mats1 (also named Mobk1b for Mps one binder kinase activator-like 1b) and Mats2 (also named Mobk11a for Mps one binder kinase activator-like 1a) (Fig. 1). Both of these two subgroups contain vertebrate mob genes from fish to mammals except for the chicken mats1, which appears to be lost in a lineage-specific manner. Fruit flies and mosquito, however, only have one mob gene in this group, which is supportive of additional gene duplication event occurring in vertebrates after their divergence from invertebrates. Interestingly, no mats gene is found in nematodes C. elegans and C. briggsae. This could be due to a lineage-specific gene loss in nematodes.

Group II contains three clusters of vertebrate mob genes but only one homolog from fly and worm (Fig. 1). Two additional mob genes in vertebrates should have resulted from two rounds of gene duplications in the common ancestor of vertebrate lineages. In these three clusters of vertebrate mob genes, however, one cluster does not have members from zebrafish and Fugu. This implies a putative gene loss from the fish lineages.

Group III, different from Group I and Group II, has only one mob gene from both invertebrates and vertebrates (Fig. 1). Interestingly, two frog and one fugu mob genes form an additional cluster with relatively long-branch lengths. Since the probability of gene losses simultaneously occurring in zebrafish, chicken and mammals is low, it is likely that fugu and frog have had some lineage-specific gene duplications. These additional frog and fugu genes might have been subject to pseudogenization, which is supported by highly diverged protein sequences as inferred from the long-branch lengths. Based on the observation of lineage-specific gene losses and/or gene duplications and the clustering of orthologs instead of paralogs, all these three groups of mob genes seem to have experienced the birth-and-death model of evolution [20]. Finally, Group IV, which contains single mob gene from each species, is considered to be the closest one to outgroup mob genes (Fig. 1). All four groups of mob genes diverged following the species tree, which is indicative of divergent evolution rather than concerted evolution.

**Exon-Intron Structures of mob Genes**

To further reveal evolutionary relationships among mob genes, we analyzed and compared exon-intron structures within the coding region of mob genes from different groups.
Fig. (1). Phylogenetic relationships of Mob family proteins. This phylogenetic tree of Mob proteins is reconstructed using neighbor-joining method with p-distances and rooted with a Mob from Diplomonadida (Giardia Lambia). One hundred forty four amino-acid sites are used with complete elimination of alignment gaps. The reliability of tree topology is tested by bootstrap method with 1000 replications. Bootstrap percentages higher than 50 are shown on interior branches. The scale bar shows 0.1 amino-acid substitution per site.
(Fig. 2). For vertebrate *mob* genes, the numbers, positions and phases of introns are generally conserved within the same group with two exceptions. One is Group III zebrafish *mob*, which has an additional intron1-2 and is lack of the intron-1, and the other one is Group IV zebrafish *mob*, whose last intron has a different position and phase from other vertebrate orthologous *mob* genes. In the central region of *mob* sequences, which corresponds to the Mob domain, Group I, II and III *mob* genes have similar exon-intron structures whereas Group IV *mob* genes are quite different and share their own unique splicing pattern. The inter-group comparison among Group I, II and III shows a higher conservation level of intron properties in the Mob domains than either amino-terminal or carboxyl-terminal regions.

**Group I:** Vertebrate *mats* genes have the same splicing pattern which produces 6 exons, while Drosophila and mosquito only have three and two exons respectively. Intron2 (phase 2) and intron3 (phase 1) have been lost in invertebrate lineages. Vertebrate *mats* genes have an additional intron+1 (phase 0) inserted in the C terminal region. Since this intron is not present in any other *mob* genes, it might be due to either a gain-of-intron in vertebrate *mats*, or, that the last intron actually arose in the common ancestor of vertebrate and invertebrate *mats* genes and then got lost in invertebrates. In addition, intron1, 2 and 3 must have been existent before the gene duplications that produced Group I and Group III *mob* genes since they are all present in most members of these two groups.

**Group II:** Different from Group I *mob* genes, invertebrate members of this group generally have more introns (five for *D. melanogaster* and six for *C. elegans*) than vertebrates (two) except for mosquito *mob2* that has one intron. The two introns from vertebrate *mob2* genes are the conserved intron 3 and an intron+1 located further downstream. Both of these two introns are shared by *C. elegans*. Immediately downstream to this intron+1, there exists Lys/Arg as the first amino acid of the flanking exon. The fly and mosquito *mob2* genes, which are lack of this conserved intron+1 in the corresponding positions, have Gln instead of Lys/Arg in the corresponding site. Coincidently, in *mats* genes presented in Fig. (2), Gln is shared by *mats* genes, from which this conserved intron+1 is absent. This might be explained by the gain-of-intron in Group II vertebrate and *C. elegans mob* genes which was triggered by the amino acid substitution from Gln to Lys/Arg. Alternatively, it could have happened via intron loss in Group I *mats* and Group II insect *mob* respectively, following the substitution from Lys/Arg to Gln. In addition to these two introns shared with vertebrates, *C. elegans mob2* has four more introns, two of which are conserved in fly *mob2* (intron-1 and intron1). Surprisingly, mosquito *mob2* only has one intron (intron+1’). This intron can be found in fly too and it might have been produced exclusively in insects. All other upstream introns have been lost in this species.

**Group III:** Group III *mob* genes remain most of the three conserved introns (intron1, 2, and 3). Intron-1 in this group is in the similar but not the same position as intron-1 in Group I. Zebrafish gained an additional intron1-2 compared to other vertebrates. Fly and mosquito both lost intron3, which is, however, the only intron from vertebrates that is present in *C. elegans* as well. Except for this intron, *C. ele-
gans mob3* has a very different exon-intron structure compared to other group III members.

**Group IV:** *mob* genes in this group are unique in their splicing patterns. They don’t have any of the three conserved introns (intron1, 2, and 3) from other three groups. Vertebrates of this group share mostly the same intron properties except that zebrafish has a different intron7. Intron1, 3, 4, 5 and 6 are shared by either some or all of the three invertebrate species. Overall, vertebrates have more introns than invertebrates in this group.

Based on the exon-intron structures of four groups of *mob* genes, we can infer the *mob* family evolution pattern in terms of gene duplications. The first gene duplication produced two *mob* genes. One evolved all the way to the contemporary Group IV *mob*. The other one duplicated again, giving rise to Group III and the common ancestor of Group I and Group II. Later on, another round of gene duplication occurred which gave birth to Group I and Group II *mob* genes. These three gene duplication events all should have occurred before the divergence of vertebrates and invertebrates since fly, mosquito and nematode homologs are all present in these four groups except that nematode *mats* is suspected to be lost during evolution. Combined with the phylogenetic tree, which shows that Giardia *mob* genes are present in almost all groups (Fig. 1), we can even trace the original three gene duplication events to the very early stage of eukaryote evolution. *mob* genes from fungi are present in Group IV, Group III and somewhere between Group I and II. This pattern also supports the order in which these four Mob groups have been established.

Both the sequence alignment and exon-intron structure showed that the N-terminal regions of Mob proteins are quite variable for the inter-group comparison but highly conserved within individual groups. Therefore, these regions have diverged more rapidly than other regions after separation of different groups. Since these regions appear to be unique to specific group, it is indicative of strong positive selection and suggests an important role of this region in group-specific functions.

**Structural Conservation of Mob Proteins**

Stavridi et al. [17] resolved the X-ray crystal structure of human Group I MOB1A/MATS1 protein and this structure has been used as a template in our analysis. Human MOB1A protein folds into a four-helix bundle core structure, which is stabilized by a Cys3His2 zinc finger holding a zinc atom in the middle. The removal of zinc using EDTA was shown to lead to the aggregation of the protein and decreases of its thermal stability [18]. On the surface of hMOB1A protein, one side is flat and rich in negative electrostatic potential. Several studies have reported that Mob proteins can interact with NDR family kinases and stimulate their kinase activity [21]. The structural analysis of NDR family kinases has identified at least two conserved basic regions, and this acidic surface of hMOB1A protein appears to be functionally important by binding with the conserved basic regions of NDR family kinases [17].

To examine how structures of four groups of Mob proteins are conserved compared to hMOB1, the program ConSurf was used to map amino acids of each group of Mob
Fig. (2). Exon-intron structures of four groups of *mob* genes. Exon-intron structures of *mob* genes are analyzed using Spidey program or directly obtained from Ensembl. Phase0 introns are indicated by open rectangles, phase1 by dark rectangles and phase2 by gray ones. Three kinds of arrow symbols (circle, triangle and rectangle) point to three categories of conserved splicing sites among intra- and inter-groups of *mob* genes. These three categories of conserved introns are assigned as intron1, 2 and 3, respectively. The flanking introns are named according to their relative positions with intron1, 2 and 3, such as minus1, 2 etc as going upstream and plus 1, 2 etc as going downstream. The numberings of introns are indicated on top of their corresponding positions unless they have the same nominations as their immediate upper sequences. The dashes with two slashes in the middle represent the unscaled sequence lengths at the ends; otherwise the lengths of all sequences are scaled based on the numbers of nucleotides in exons.
proteins and colored amino acid residues according to their conservation levels by using the structure of hMOB1 as template (Fig. 3). Overall, Mob proteins are highly conserved in their amino acids sequences, among which Group I members have the highest conservation level as expected (Fig. 3A). Group II, although not as conserved as Group I, preserve high sequence similarity in the C-terminal of H2, C-terminal of H5, and four residues of the Cys2His2 structure (Fig. 3B). Group III and Group IV Mobs are both conserved in their zinc finger regions and the flanking structural elements, including N terminal of H4, C-terminal of H5 and some residues from H2 (Fig. 3C, D). Thus, comparison of all four groups of Mob proteins uncovered the highly conserved zinc finger structure and its closely positioned motifs, and also some residues that are critical for stabilizing loop structures.

To identify residues that are highly conserved among different groups, human, mouse, zebrafish and fly Mob proteins were used to make alignments (Fig. 4). A total of seven residues are conserved in all four groups of vertebrate and fly Mobs. Pro48 from L1 and Pro133 from L2 may play an important role in stabilizing the loop structure and further maintaining the folding of core four-helix bundle. Moreover, the N-terminals of H2 and H4 helices have the conserved Trp56, Ala111 and Tyr114, all of which are hydrophobic residues. Trp56 is shown to have buried side chains [17]. Ala111 and Tyr114 are spatially close to zinc finger motif and facing inward to the core of helix bundle, which may contribute to the protein stability by hydrophobic interactions. Two remaining conserved residues are Cys79 and Cys84. These two cysteins, together with His161 and His166, coordinate a zinc atom. His161 and His 166 are also highly conserved with the exception of zebrafish Mob4, in which these two histidines are both replaced by isoleucines. The ubiquitous presence of this Cys2His2 zinc finger indicates that it is critical structural feature for Mob family proteins.

**Fig. (3).** Structural conservation of Mob proteins. Amino acids of Mob proteins are mapped according to their evolutionary conservation onto the three-dimensional structure of human MOB1A using program Consurf. (A-D) Comparison of group I-IV Mob proteins. (E) Comparison of all groups of mob proteins.
Fig. (4). Sequence alignment of vertebrate and fly mob proteins. Human, mouse, zebrafish and fly Mob proteins were used to make profile alignments and the sequences cover residues 33 to 206 of human MOB1, in which the main motifs of the crystal structure of hMOB1A/hMATS1 protein were displayed (Stavridi et al. 2003). The sequences analyzed here cover the entire Mob domain. The protein names follow the nomenclature described in Table 1. We use dots for conserved residues and dashes for alignment gaps. The numbers on top of the alignment blocks reflect the actual amino acid positions of hMATS1. Asterisks are used to locate conserved residues for all aligned sequences. The secondary structures of hMATS1 are shown as: green bar-helix, brown arrow-beta sheet, blue parenthesis-loop. The fixed amino acid substitutions are highlighted by white fonts in black background (Group I vs Group II), red fonts in blue background (Groups I/II vs Group III), and orange fonts in gray background (Groups I/II/III vs Group IV). The properties of these substitutions are indicated on top of the corresponding sites, R for radical substitution and C for conservative substitution. The amino acid substitutions encoded by S. cerevisiae mob1 mutant alleles (Luca and Winey 1998, Stavridi et al. 2003) are positioned to the corresponding sites at the bottom of alignment blocks. The numbers used for the mutants’ names are the actual residue positions of yeast Mob1 protein. The letters flanking the left and right sides of these numbers indicate the amino acids before and after mutations, respectively.
Divergence of Mob Proteins

Structural diversification would allow functional divergence of Mob proteins. Group I and group II have 22 fixed substitutions, most of which are located in helix regions (19 out of 22) (Fig. 4). Radical substitutions in helices are distributed in H2, H5 and H7 with the numbers of 3, 4 and 2 for each. Based on the phylogeny relationships of mob genes, which indicate that Groups I and II were produced following Group IV and Group III, His60 was suspected to have been replaced by Asn in the common ancestral sequence of Mob1, Mob2 and Mob3 and then after the duplication that generated Mob1 and Mob2, the relaxed functional constraints on one of the two duplicates (Mob2 in this case) made the backward mutation (N→H) possible. The neighboring residue Thr61 might have been subject to the similar evolution course, where Mob1 and most Mob3 share Thr while Mob2 and Mob4 contain conservative residues, Val and Leu, respectively. The third radical substitution (Q67R), however, is different from the previous two in that Mob2 and Mob3 are now sharing the conservative positively charged amino acids, Arg and His, but Mob1 and Mob4 have Gln and Glu instead. The substitution of T150K from H5 happened in the same way as Q67R, where Mob1/Mob4 has Thr/Ser but Mob2 and Mob3 share Lys. H164I and Q167R are next to each other and these two sites function to position H5 and H6 together in a perpendicular direction. The interaction between these two residues might be required to maintain this particular angle. Based on the fact that these two sites always carry the opposite electrostatic charges and even if radical substitutions occurred, they occurred in a compensatory way (I→H, H→Q), the electrostatic interaction between them is very likely to be critical for the structural integrity. The Ala160V substitution, although it is radical, might not have dramatic effect since both Ala and Val are neutral and only differ in their volume. The last two radical changes in helices are located in the middle region of H7 and both of them come together with the flanking conservative F-to-Y substitution. The only radical substitution (C109L) in non-helix region is located N terminally to H4. Since Cys is generally conserved in Groups I, III and IV, the presence of Leu in Group II is supposed to have arisen in a group-specific way and might be critical for Group II specificity.

Between Groups I/II and Group III, there are totally seven fixed amino acid substitutions in the Mob domain and five of them are radical changes (Fig. 4). Among these five changes, two are located in the non-helix regions, which might be critical for group-specific functions. P106K, E176H and L201T could be due to substitutions specifically occurring in Group III since the rest three groups share the same amino acids. P139E, however, might be the result of two independent substitutions in Groups I/II (P) and Group III (E) from their ancestral gene, or Glu is the ancestral residue and Pro has derived from it. The last radical change is V59S from H2. This region has four continuous radical substitutions (site 58-61), which cover all three categories. It may indicate the importance of this region in the functional differentiation of different groups of Mob proteins. For two conservative substitutions (L118V, R157H), both occur in helix regions and may have less effect.

The comparison between Groups I/II/III and Group IV identifies four radical substitutions and eight conservative ones (Fig. 4). The radical changes are mainly limited to non-helix regions (3 out of 4). The only radical substitution on helix is A58Y from H2. This residue Ala was replaced by Ile in one of the yeast conditional mob1 mutants (mob1-95) [1]. Thus, this Ala might be critical for some specific function shared by Groups I, II and III but not by Group IV. The rest three radical changes are located C terminally to the two beta strands (W179C, D199A) and N terminally to H5 (F140E). Phe140 was thought to be involved in structural interaction, which together with Phe132 and Phe144 form hydrophobic interactions with each other and also with conserved Ile151 from H5 [17].

A number of mutations in the yeast mob1 genes have been molecularly characterized, most of which are located in H2 helix, especially in its N-terminal, and also L1 loop (STAVRIDI et al. 2003). The other ones are scattered on H4, C-terminal of H5 and N-terminal of H7. Also some are residing in the non-helix region that connects H2 and H3. The mutations of E151K (mob1-77), Q167R (mob1-55) and Y193H (mob1-55) can cause late mitotic arrest and cytokinesis defects although these residues are only conserved in Group I and/or Group II Mobs but not in Group III ones. It suggests that Mob1 might have distinct function from Group III Mobs or have acquired new mechanism that makes these residues essential for Mob1 but not for Mob3. The other mob1 alleles mostly have mutations occurring in conservative residues shared by Mob1 and Mob3, indicating their significant role in the common features of Group I and Group III Mobs.
Human mob Genes are Generally Expressed in Most Tissues and During Development

At present time, very little is known about the functions of most mob family genes. Analysis of mob gene expression would help understand how mob genes might be functionally required in different tissues during development. For this reason, we have focused on human MOB genes to elucidate their expression profiles since abundant Expression Sequence Tag (EST) data is available from UniGene database in NCBI. Overall expression of hMATS1 during development is the highest among all hMOB genes and is almost comparable to that of control gene, H1 histone member 0 (H1_0) (Fig. 5A). Human MOB genes are generally expressed throughout development except that some members show zero EST in neonate, infant or juvenile stage (Fig. 5A). Considering that there are only 5x10^4 or less total ESTs from each of these three stages, the failure to detect MOB-related ESTs might not accurately reflect expression of the MOB genes.

To analyze human MOB expression in different tissues, only the tissues that have more than 10^5 of total ESTs were used. Generally, all hMOB genes are expressed in most tis-

![Graph A](image1)

**Fig. (5). Expression levels of human MOB genes in different tissues.** The transcript levels of human MOB genes were obtained from EST profiles of UniGene in NCBI. Y axis represents the number of transcripts per million (TPM). X axis shows the control gene (H1 histone member 0) and all seven human MOB genes. Tissues and developmental stages are assigned with different colors. (A) Breakdown by developmental stages displays the expression levels of H1_0 and human MOB genes in embryoid body, blastocyst, fetus, neonate, infant, juvenile and adult stages. Numbers of ESTs from neonate and infant are below 5x10^4. There are more than 5x10^4 ESTs analyzed for other stages (Table S2). (B) Breakdown by tissues represents expression levels of H1_0 and human MOB genes in 18 representative tissues. There are at least 10^5 ESTs analyzed for each of the 18 tissues (Table S2).
sues at levels lower than H1.0 gene (Fig. 5B). Among the seven hMOB genes, hMATS1 has the highest average expression. hMOB2A has outstanding expression in blood, which is five times higher than its average level. All other hMOB genes, however, are mostly expressed within two times of their average levels. Expression of hMATS2, hMOB2B and hMOB4 in kidney and expression of hMATS2, hMOB2C and hMOB3 in pancreas are more than twice higher than their average levels. Moreover, placenta, eye and prostate show more than twice higher expression levels for hMOB2B, hMOB2C and hMOB3, respectively. Interestingly, some tissues show no expression for some of the hMOB genes, such as hMATS2 in intestine and testis, hMOB2B and hMOB3 in muscle, as well as hMOB2A and hMOB2C in ovary. Thus, while hMOB genes are generally expressed in most tissues, some of them appear to be preferentially expressed in certain tissues.

DISCUSSION

The mob Gene Family Has Four Distinct Groups

Up to date, there are more than 270 mob genes identified from eukaryotes. Through a molecular evolutionary approach, we have elucidated evolutionary relationships among these mob genes. It is clear that there are four distinct groups of mob genes (Groups I-IV), which should have evolved before the divergence of vertebrate and invertebrate animals. A similar conclusion was reached in a previous evolutionary analysis done with 192 mob genes [22]. Like other gene families, gene duplication provided a mechanism for generating new family members. Because protists have mob genes in almost all groups, the first three gene duplication events should have occurred at a very early stage of eu- karyotic evolution. As mob gene is only found in eukaryotes, the mob genes should have arisen after the divergence of prokaryotes and eukaryotes and the mob gene family appears to be an innovation of eukaryotic organisms.

Conserved Features of Mob Proteins

Mob proteins appear to share the following three major structural features. The first feature is that Mob has an atypical Cys2-His2 motif responsible for zinc binding [17]. This is a general property of all Mob proteins probably with an exception of zebrafish Mob4 (Fig. 4). The second feature is that there is a flat surface on one side of Mob protein rich in acidic residues. This structure is presumably critical for Mob1 to interact with its partner such as NDR family protein kinases. The third feature is that generally Mob proteins are small in size. In addition to the Mob domain, there are no other obvious domains in Mob proteins. While it is common for a protein domain to be linked with other domains in a protein to increase structural and functional complexity, it is not clear why Mob proteins are restricted to increase their size and not allowed to combine with other protein domains. As Mob protein can associate with other proteins such as Ndr kinase, it is possible that there is a space constriction for Mob to fit into a protein complex, which prevents Mob from altering its overall size.

Since mob genes are so highly conserved, they are expected to play important roles in establishing and maintaining key features of eukaryotes during evolution. In one case, genetic analysis has shown that Drosophila mats gene plays an essential role in cell proliferation and apoptotic control during tissue growth and a human MATS gene can functionally replace fly mats [12]. At the molecular level, Mats functions as a binding partner and coactivator of Wts/Lats protein kinase [12]. Since the Drosophila Mats protein has been shown to function as a growth inhibitor to control tissue growth during development, we speculate that loss of MATS function might promote tumorigenesis of human cancers. Furthermore, activation of Ndr family kinases by Mob has been demonstrated in wide variety of species from yeast to humans [reviewed in 21]. Importantly, yeast Mob2 also binds NDR family protein kinases, Cbk1 and Orb6, to control polarized cell growth [reviewed in 21]. Therefore, this conserved function for Mob as kinase coactivator can be at least traced back to the time when the first mob gene duplication occurred. Investigation of whether Group IV Mob can function as a kinase activator would help investigate the possibility of this molecular feature being innovated at the very beginning of mob gene evolution.

Functional Diversification of Mob Proteins

Structural alterations make it possible for functional diversification. To characterize structural differences among four groups of Mob proteins, both radical and conservative substitutions between different groups have been examined. As summarized in Table 2, both types of substitutions can occur in helix as well as non-helix regions (Fig. 4). Moreover, substitutions occurred throughout Mob protein from the amino to carboxyl termini. In one example, Thr74 is a conserved residue among Group I/II/III Mob proteins with the exception of zebrafish Mob3, but it is replaced by a Lys in Group IV (Fig. 4). This residue was shown to be phosphorylated by MST2 protein kinase and important for MOB1 to activate NDR1 protein kinase [23]. Through this mechanism, MST2 protein kinase functions as an upstream regulator of Group I/II/III but not Group IV Mob proteins. As we begin to understand the importance of phosphorylation for Mob regulation [23-25], conserved sequence changes would allow Mob proteins of various groups to be differently regulated by phosphorylation and other protein modification mechanisms.

Table 2. The Number of Fixed Substitutions Between Groups of Representative mob Proteins

<table>
<thead>
<tr>
<th>Region</th>
<th>Radical</th>
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<td>9</td>
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<td>Non-helix</td>
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<tr>
<td>I &amp; II vs III</td>
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<td>3</td>
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<td></td>
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<td>I &amp; II &amp; III vs IV</td>
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<td>1</td>
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<tr>
<td></td>
<td>Non-helix</td>
<td>3</td>
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SUPPLEMENTARY MATERIAL

This article also contains supplementary data and it can be viewed at www.bentham.org/open/tocellsj

ABBREVIATIONS

EST = Expressed Sequence Tag
Lats = Large tumor suppressor
Mats = Mob as tumor suppressor
Mob = Mps one binder
MST = Mammalian Sterile20-like protein kinase
NDR = Nuclear Dbf2-related protein kinase
Wts = Warts

REFERENCES


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TEAD mediates YAP-dependent gene induction and growth control

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TEAD mediates YAP-dependent gene induction and growth control

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The YAP transcription coactivator has been implicated as an oncogene and is amplified in human cancers. Recent studies have established that YAP is phosphorylated and inhibited by the Hippo tumor suppressor pathway. Here we demonstrate that the TEAD family transcription factors are essential in mediating YAP-dependent gene expression. TEAD is also required for YAP-induced cell growth, oncogenic transformation, and epithelial–mesenchymal transition. CTGF is identified as a direct YAP target gene important for cell growth. Moreover, the functional relationship between YAP and TEAD is conserved in Drosophila Yki (the YAP homolog) and Scalloped (the TEAD homolog). Our study reveals TEAD as a new component in the Hippo pathway playing essential roles in mediating biological functions of YAP.

Keywords: TEAD; YAP; CTGF; Hippo; transcription, cancer

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lished the importance of the Hippo pathway in human cancer.

Several transcription factors, including ErbB4, Runx2, TEAD, and p73, have been reported to interact with YAP (Yagi et al. 1999; Vassilev et al. 2001; Basu et al. 2003; Komuro et al. 2003). However, the significance of these transcription factors in mediating the biological functions of YAP, especially in promoting cell growth, has not been demonstrated. In this study, we identified TEAD as the most potent YAP target from a transcription activity-based screen. By means of dominant-negative or RNAi, we further showed that TEAD is required for YAP to stimulate gene expression, cell growth, anchorage-independent growth, and EMT. We identified the connective tissue growth factor (CTGF) as a direct target gene of YAP and TEAD. Interestingly, knockdown of CTGF blocks YAP-stimulated cell growth and significantly reduces YAP-induced colony formation in soft agar. Furthermore, experiments in Drosophila demonstrated that Sd and Yki genetically interact to enhance tissue growth and organ size. Together, our observations establish TEAD as the key transcription factor in the Hippo pathway acting downstream from YAP.

Results

**TEAD mediates YAP-dependent gene induction**

To identify YAP target transcription factors, we screened a human transcription factor library in which the known or putative transcription factors were fused to Gal4 DNA-binding domain. Clones of the Gal4-TF library (a total of 1100) (J.D. Lin, unpubl.) were individually cotransfected with a 5× UAS-luciferase reporter, which is driven by five Gal4-binding elements, in the presence or absence of YAP cotransfection. This unbiased strategy identified TEAD2, TEAD3, and TEAD4 as the strongest positives based on the transcription reporter assay. The human genome contains four TEAD transcription factors. TEAD1 was not present in our Gal4-TF library, but it could also be potently activated by YAP [Fig. 1A]. Several other transcription factors, including ErbB4 and RUNX2, have been reported to interact with YAP (Yagi et al. 1999; Komuro et al. 2003). However, the activation of ErbB4 by YAP is much weaker than that of TEAD [Fig. 1A]. Furthermore, YAP showed a strong physical interaction with TEAD but little interaction with RUNX2 [data not shown]. These data indicate that the TEADs may represent the major target transcription factors of YAP.

By point mutation scanning, we found that the YAP Ser 94 to alanine (S94A) mutant was defective in TEAD4 activation [Fig. 1B] as well as other TEADs activation [data not shown]. However, YAP-S94A retains full potential to activate RUNX2 [Fig. 1B] and ErbB4 [data not shown]. This indicates that mutation of YAP S94 selectively abolishes its ability to activate TEAD but does not impair its general transcriptional activity. Consistently, we observed that YAP-S94A lost its ability to physically interact with TEAD4 [Fig. 1C] and other TEADs [data not shown]. To assess the importance of TEAD interaction in YAP-induced gene expression, we established MCF10A stable pools with expression of YAP, constitutively active YAP-5SA [Zhao et al. 2007], and YAP-S94A. Gene expression profiles were determined by microarray (Supplemental Table S1). Our data showed that YAP-5SA caused a stronger induction of YAP-inducible genes than the wild-type YAP [Fig. 1D]. Interestingly, YAP-S94A was severely compromised in gene regulation [both induction and repression] [Fig. 1D; Supplemental Table S1]. We reported previously that YAP regulates gene expression in NIH-3T3 cells [Zhao et al. 2007]. Comparing the data from NIH-3T3 and MCF10A cells by Gene Set Enrichment Analysis (GSEA) [Subramanian et al. 2005], we found a significant overlap of gene profiles between the two cell lines [Supplemental Fig. S1A]. The majority of genes that are affected by YAP expression are similarly regulated (either up or down) in both NIH-3T3 and MCF10A cells [Supplemental Table S2], while a subset of genes is oppositely regulated in NIH-3T3 and MCF10A cells [Supplemental Table S2].

Among the confirmed YAP-inducible genes in MCF10A were CTGF and ITGB2 [integrin β2]. They were strongly induced by YAP-5SA but not by YAP-S94A [Supplemental Fig. S1B]. Furthermore, coexpression of the dominant-negative TEAD1-ΔC, which has a deletion of the C-terminal YAP-binding domain, blocked the induction of both CTGF and ITGB2 [Supplemental Fig. S1B]. The four TEAD family members are all expressed in MCF10A cells, while TEAD1 has the highest expression [data not shown]. We generated lentiviral constructs with shRNAs designed in a region identical in TEAD1, TEAD3, and TEAD4. Indeed, these shRNAs were able to knock down TEAD1, TEAD3, and TEAD4 concurrently but not TEAD2 [Supplemental Fig. S1C]. Nevertheless, these TEAD1/3/4 shRNAs strongly blocked the induction of CTGF and ITGB2 by YAP-5SA expression [Fig. 1E]. These data demonstrate that in MCF10A cells, the TEAD1/3/4 transcription factors play a critical role in the expression of YAP-dependent genes.

If TEAD plays a major role in YAP-regulated gene expression, they should occupy a similar set of gene promoters. We performed genome-wide location analysis of YAP and TEAD1 occupancy in MCF10A cells by chromatin immunoprecipitation (ChIP)-on-chip experiments. Interestingly, our results demonstrated that YAP and TEAD1 co-occupy >80% of the promoters pulled down by either of them [Fig. 1F; raw data in Supplemental Table S3]. The Androgen Receptor [AR]-associated genes were included as a control, which showed a much lesser degree of overlap with those occupied by YAP compared with TEAD1 [odds ratio = 34.6, \( P < 0.00001 \)]. This observation further supports that the overlap between YAP and TEAD1 targets is not a random event. Gene Set Enrichment Analysis (GSEA) demonstrated that a significant \( P < 0.001 \) portion of YAP-bound genes are differentially expressed upon YAP overexpression in MCF10A cells. Since YAP does not have DNA-binding activity, these data strongly indicate that TEAD plays a major role in mediating the binding of YAP to gene promoters.
Figure 1. TEAD is required for YAP-induced gene expression. (A) YAP potently activates TEAD family transcription factors. The indicated Gal4-fused transcription factors were cotransfected with a 5x UAS-Luc reporter and a CMV-β-gal construct into 293T cells in the presence or absence of YAP. The β-galactosidase activity normalized luciferase activity in the absence of YAP (Gal4-TEAD1 in the absence of YAP in the left panel) was set to 1. Flag-YAP Western blot shows that the YAP expression level was not decreased by ErbB4. (B) YAP-S94A cannot activate TEAD4. The indicated plasmids were cotransfected with a 5x UAS-luciferase reporter for Gal4-TEAD4 or a 6x OSE2-luciferase reporter for RUNX2 into 293T cells. Luciferase activity was measured and normalized to cotransfected β-galactosidase. (C) Serine 94 of YAP is required for its interaction with TEAD4. The indicated plasmids were transfected into HEK293 cells. Flag-YAP (left panel) or Myc-TEAD4 (right panel) was immunoprecipitated, and the immunoprecipitates were probed as indicated. (D) YAP-S94A is defective in gene expression regulation. The left panel shows cluster analysis of gene expression profiles in YAP-WT, 5SA, or S94A-overexpressing MCF10A cells. The group of genes presented was chosen by the following standard: a P call in all samples and up-regulated more than fivefold or down-regulated more than fourfold by YAP-wild-type overexpression. Cluster analysis was done with Eisen Lab Cluster software using average linkage clustering. (Right panel) The same data sets were drawn into boxplots using the R program. Red and green indicate up-regulated and down-regulated genes, respectively. (E) TEAD is required for YAP-induced expression of CTGF and ITGB2. The indicated shRNAs were infected into native or YAP-5SA-expressing MCF10A cells. Expression of CTGF and ITGB2 were determined by quantitative RT–PCR and compared to vector control cells. (F) Scramble shRNA control, #1 and #2 two different shRNAs targeting TEAD1/#3. (F) YAP and TEAD1 occupy common promoters. ChIP-on-chip was performed with YAP or TEAD1 antibody against endogenous proteins in MCF10A cells. Genome-wide location analysis was performed. AR ChIP was included as a negative control.
TEAD binding is required for YAP-induced cell growth and EMT

We reported that YAP expression in NIH-3T3 cells enhances cell growth (Zhao et al. 2007). NIH-3T3 stable pools with expression of YAP and YAP-S94A were established, and cell growth was determined. We found that YAP-S94A was much less potent than the wild-type YAP to stimulate NIH-3T3 cell growth (Fig. 2A). Furthermore, in MCF10A cells, wild-type YAP induced cell proliferation even when cells reached confluency, while the YAP-S94A mutant was largely inactive as determined by the staining of proliferation marker Ki67 [Supplemental Fig. S2A]. To confirm that the loss of growth-promoting activity in YAP-S94A is due to the loss of its interaction with TEAD, we generated a TEAD1-YAP-S94A fusion protein. Interestingly, this fusion protein stimulated NIH-3T3 cell growth as effectively as the wild-type YAP, while neither TEAD1 nor YAP-S94A stimulated cell growth (Fig. 2A). Furthermore, the TEAD1-YAP-S94A fusion also rescued the expression of Ctgf and Inhba, two YAP target genes, in NIH-3T3 cells (Fig. 2B). We also examined the effect of S94A mutation in the constitutively active YAP-5SA background in MCF10A cells. Expression of YAP-5SA resulted in the formation of much larger acini in three-dimensional (3D) culture compared with vector control. Importantly, this effect was largely reduced if an S94A mutation was introduced into YAP-5SA (Fig. 2C). These results indicate that S94, hence TEAD binding, is required for YAP-induced cell proliferation.

It has been reported that YAP induces EMT in MCF10A cells (Overholtzer et al. 2006). Indeed, expression of the active YAP-5SA induced EMT-like morphological change in monolayer culture (Fig. 2C). However, YAP-5SA-S94A was not effective in eliciting EMT morphology. Furthermore, in 3D culture, YAP-5SA-S94A failed to induce complex-shaped large acini with spike-like projections and rough surface, which were obvious in YAP-5SA-expressing cultures (Fig. 2C). As another hallmark of EMT, YAP-5SA-expressing cells also displayed disorganized adherens junctions, as shown by the loss of cell–cell junction localized E-cadherin, and the switch from cortical actin to stress fibers (Fig. 2D). However, these phenotypes were not seen in YAP-5SA-S94A-expressing cells. YAP-5SA expression also changed the expression pattern of epithelial and mesenchymal markers, which was not induced by YAP-5SA-S94A expression (Fig. 2E). These results indicate that S94 of YAP, presumably by mediating TEAD interaction, is at least partially responsible for YAP function in inducing EMT.

To further confirm the function of TEAD, we used shRNAs to knock down TEAD1/3/4 in YAP-5SA-expressing cells. TEAD1/3/4 knockdown not only reversed the EMT-like morphology in monolayer and 3D cultures, but also rescued the expression of epithelial markers (Fig. 2F; Supplemental Fig. S2B). Knockdown of TEAD1/3/4 also significantly shrank the aberrantly enlarged acini caused by YAP-5SA expression, further supporting a role of TEAD in YAP-induced growth. A YAP-dependent function of TEAD in cell growth is also implicated in Sveinsson’s chorioretinal atrophy, a rare genetic disease caused by TEAD1 mutation and characterized by atrophic lesions involving retina and choroids (Fossdal et al. 2004; Kitagawa 2007). The mutated tyrosine Y406 is highly conserved in TEAD family members (Supplemental Fig. S2C), and is located within the YAP-binding domain [Supplemental Fig. S2D]. Interestingly, mutation of this tyrosine residue in TEADs abolished their interaction with and their activation by YAP (Supplemental Fig. S2E–G), which may explain the atrophic phenotype caused by this mutation.

Anchorage-independent growth is a hallmark of oncogenic transformation. YAP overexpression is reported to induce anchorage-independent growth of MCF10A cells (Overholtzer et al. 2006). We observed that YAP-5SA potently induced MCF10A colony formation in soft agar. In contrast, YAP-5SA-S94A was unable to induce anchorage-independent growth of MCF10A cells (Fig. 2G; Supplemental Fig. S2H). Similarly, almost no colony was formed if TEAD1/3/4 were down-regulated in the YAP-5SA expressing cells (Fig. 2G; Supplemental Fig. S2H). These data indicate the requirement of at least one of TEAD1/3/4 for the YAP-induced anchorage-independent growth. Together, the above observations support a model in which TEAD is essential for the function of YAP in cell proliferation, EMT, and oncogenic transformation.

CTGF is a direct YAP-TEAD target gene required for cell growth

YAP expression affected many cell proliferation-related genes [Supplemental Table S1]. However, cyclin E and IAP, the key Yki-inducible genes in Drosophila, were not significantly induced by YAP in either NIH-3T3 or MCF10A cells [Supplemental Table S1]. This indicates that there might be different genes in mammalian cells to mediate YAP function. CTGF is highly induced by YAP expression in both NIH-3T3 and MCF10A cells, and its promoter is co-occupied by YAP and TEAD1, as shown by ChIP (Fig. 3A); therefore, it might be a direct YAP target gene. We cloned the CTGF promoter into a basic luciferase reporter and found that it was potently activated by YAP but not by YAP-S94A, and the activation was further enhanced by TEAD1 coexpression (Fig. 3B). Expression of the dominant-negative TEAD1-ΔC, but not the TEAD1-ΔC-AD [in which the C-terminal YAP-binding domain was replaced by the YAP transactivation domain], blocked the activation of CTGF reporter by YAP (Fig. 3C). These results indicate that YAP activates the CTGF promoter through TEAD. Examination of the CTGF promoter region revealed three putative TEAD-binding sites (Fig. 3D; Anbanandam et al. 2006). Individual or combinatorial mutation of the putative TEAD-binding sites indicated that TB2 and TB3 were more important for CTGF promoter activity while TB1 was also involved (Fig. 3E).

The function of endogenous YAP and TEAD in CTGF expression was examined by YAP or TEAD1/3/4 knock-
Figure 2. TEAD is required for YAP activity in growth promotion and EMT. (A) YAP-S94A is defective in promoting cell growth. The growth curve of NIH-3T3 stable cells with expression of Vector, YAP, YAP-S94A, TEAD1, or TEAD1-YAP-S94A was determined. (B) Fusion of YAP-S94A with TEAD1 rescued YAP target gene expression. (Right panel) NIH-3T3 stable cells with expression of YAP-S94A, TEAD1, and TEAD1-YAP-S94A fusion protein were generated, and the expression of these proteins was shown by anti-Myc-tag Western blot. The expression of Ctgf and Inhba, two YAP target genes in NIH-3T3 cells, was measured by quantitative PCR. The induction of these two genes by YAP-WT was also shown for comparison. (C) YAP-5SA-S94A is compromised in eliciting EMT-like morphology. Indicated MCF10A stable cells were cultured in monolayer or in 3D on reconstituted basement membrane for 16 d before pictures were taken. (D) YAP-5SA-S94A is defective in reducing membrane E-cadherin and cortical actin. The indicated MCF10A stable cells were stained by anti-E-cadherin (green), rhodamine-phalloidin (red), and DAPI (blue). (E) The TEAD-binding-defective YAP is compromised in altering EMT marker expression. Western blot of epithelial and mesenchymal markers was performed using lysates from indicated MCF10A stable cells. (F) TEAD1/3/4 shRNAs blocked YAP induced EMT-like morphology and acinar overgrowth. YAP-5SA-expressing MCF10A cells were infected with indicated shRNA lentiviruses. The morphology in 2D and 3D culture was documented as in C. (G) TEAD1/3/4 shRNAs blocked YAP-induced anchorage-independent growth in soft agar. The indicated MCF10A stable cells were plated in soft agar and allowed to grow for 3 wk, after which colonies were stained with crystal violet and counted.
Figure 3. CTGF is a direct target of YAP and TEAD. (A) Both YAP and TEAD1 bind to the CTGF promoter. ChIP from MCF10A cells was performed with control IgG, YAP, or TEAD1 antibody as indicated. The presence of CTGF promoter was detected by PCR. (B) Activation of CTGF reporter by YAP and TEAD1. A luciferase reporter driven by CTGF promoter was cotransfected with YAP wild type or S94A mutant as indicated with or without TEAD1 cotransfection. Luciferase activity was measured and normalized to cotransfected β-galactosidase. (C) Dominant-negative TEAD1 blocks the YAP stimulation of the CTGF reporter. The indicated plasmids were cotransfected, and luciferase activity was determined as in B. (D) The human CTGF promoter region contains three putative TEAD-binding sites. The putative TEAD-binding sites (TB1–TB3) are shown in red. (E) The putative TEAD-binding sites are important for CTGF promoter activity. The putative TEAD-binding sites (TB) were mutated individually or in combination. The luciferase activity of each reporter was measured in the presence or absence of YAP and TEAD1. The activation folds by YAP and TEAD1 are shown. (F) YAP and TEAD are required for CTGF expression. ACHN cells were infected with the indicated shRNA lentiviruses, and CTGF mRNA levels were determined by quantitative RT–PCR. (G) Knockdown of YAP or TEAD1/3/4 decreases CTGF protein levels. Experiments were similar to F except Western blotting was performed with the indicated antibodies. (H) YAP, TEAD, and CTGF are important for the AHCN cell growth. YAP, TEAD1/3/4, and CTGF were knocked down by shRNAs. Cell growth rate was determined. (I) CTGF knockdown attenuates YAP induced anchorage-independent growth in soft agar. The indicated MCF10A stable cells were plated in soft agar and allowed to grow for 3 wk, after which colonies were stained with crystal violet and counted. Pictures of the stained colonies were presented in higher magnification to show the colony size reduction by CTGF shRNAs.
down in ACHN cells, which have elevated YAP activity due to a mutation of Sav, a key component of the Hippo pathway (Tapon et al. 2002). RNAi specificity and efficiency were confirmed by quantitative RT–PCR [Supplemental Fig. S3A] and Western blot [Fig. 3G]. We found that knockdown of either YAP or TEAD1/3/4 caused a dramatic reduction of both CTGF mRNA [Fig. 3F] and protein [Fig. 3G]. We next examined the function of CTGF in mediating the cellular function of YAP. Similar to the knockdown of YAP and TEAD1/3/4, knockdown of CTGF significantly inhibited ACHN cell growth [Fig. 3H]. These data further demonstrate the functional significance of TEAD1/3/4 and CTGF as important downstream targets of YAP in the Hippo pathway in cell growth regulation. Furthermore, knockdown of CTGF in the YAP-S5A-expressing MCF10A cells decreased the acini growth and reversed the complex-shaped and rough surface morphology in 3D culture [Fig. 3I]. However, CTGF knockdown did not reverse the EMT-like morphology in monolayer culture. These results indicate that CTGF plays an important role in the growth-promoting function but may not be required for the EMT-inducing activity of YAP.

We also tested the effect of CTGF knockdown in the anchorage-independent growth potential of YAP-S5A-overexpressing MCF10A cells. Although CTGF knockdown did not completely block the anchorage-independent growth of YAP-S5A-overexpressing MCF10A cells, it significantly decreased the number of colonies formed [Fig. 3J; Supplemental Fig. S3B] and dramatically reduced the colony size [Fig. 3J]. However, expression of CTGF alone did not phenocopy the effects of YAP overexpression in MCF10A cells [data not shown]. Therefore, we speculate that CTGF works with other YAP target genes to mediate the oncogenic transformation potential of YAP.

YAP/Yki and TEAD/Sd genetically interact to promote tissue growth in Drosophila

To investigate the function of TEAD in YAP-induced growth control, we generated transgenic flies that express human YAP-S127A [an active form] or YAP-S94A/S127A in developing eyes. YAP-S127A overexpression significantly increased eye size [Supplemental Fig. S4A, panels a,d] and the number of interommatidial cells [Fig. 4A, panels a,d]. Mutation of S94A dramatically decreased the activity of YAP-S127A in promoting tissue growth [Fig. 4A, panel e; Supplemental Fig. S4A, panel e]. Scalloped [Sd] is the only TEAD homolog in Drosophila. We found that Yki directly interacted with Sd in an in vitro binding assay [Supplemental Fig. S4B]. Furthermore, Yki S97A mutation [equivalent to YAP-S94A] diminished its interaction with Sd. Moreover, this Sd-binding-defective Yki-S97A mutant was less potent in stimulating growth in vivo compared with wild-type Yki [Fig. 4A, panels a–c; Supplemental Fig. S4A, panels a–c]. The functional defect of the TEAD-binding-deficient YAP/Yki was further confirmed by generating overexpression flip-out clones in the Drosophila larval wing discs as labeled by positive GFP expression [Fig. 4B]. Both YAP-S127A and Yki are potent in stimulating tissue growth as individual clones, and the whole discs were generally larger than wild-type clones or discs [Fig. 4B, panels a,b,d]. However, neither YAP-S94A/S127A nor Yki-S97A showed a similar level of growth-promoting effect [Fig. 4B, panels c,e]. These data indicate that TEAD/Sd binding is important for the physiological function of YAP/Yki.

We next tested the genetic interaction between Yki and Sd. A strong loss-of-function allele of sd dominantly suppressed the enlarged and rough eye phenotypes caused by Yki overexpression [Fig. 4C, panels a–d]. Thus, the level of Sd is critical for Yki to promote tissue growth. Overexpression of Sd caused small eyes [Fig. 4C, panel e], presumably due to a dominant-negative effect (Simmonds et al. 1998), but it did not result in lethality. This phenotype was strongly enhanced by reduction of yki levels, such that all of these flies died at the late pupal stage and had no eyes [Fig. 4C, panel f]. Furthermore, coexpression of Yki with Sd suppressed the reduced eye phenotype caused by Sd overexpression [Fig. 4C, panels e,g,h]. In fact, the eyes of animals overexpressing both Yki and Sd were enlarged more than those of animals that only expressed Yki. Therefore, Sd overexpression enhanced the Yki overexpression phenotypes. Together, these results indicate that Sd is a critical functional partner of Yki, a conclusion consistent with TEAD as a critical downstream target transcription factor of YAP.

Discussion

The Hippo pathway plays an important role in the regulation of cell and tissue growth (Saucedo and Edgar 2007). Dysregulation of this pathway, such as mutations in NF2, leads to human cancer (McClatchey et al. 1998). Acting at the end of the Hippo pathway is the YAP transcription coactivator, which is an oncogene capable of promoting cell growth, oncogenic transformation, and EMT in cultured cells. YAP overexpression increases organ size and causes cancer in transgenic mice (Dong et al. 2007). An important open question in the field is the transcription factor(s) that mediate the biological function of YAP. In this study, we demonstrated that the TEAD family transcription factors play an essential role in YAP-dependent gene expression and cell growth stimulation. The functional relationship between YAP and TEAD is conserved in Drosophila, in which Yki acts through Sd to regulate cell growth and organ size. During the preparation of this manuscript, it was reported that Sd mediates Hippo signaling downstream from Yki (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008). These Drosophila studies are completely consistent with our Drosophila data and further support our conclusion that TEAD is a key transcription factor mediating YAP function in mammals.

Although both Yki and YAP promote cell and tissue growth in Drosophila and mammals, respectively, the genes induced by these two transcription coactivators are not identical. For example, cyclin E is induced by Yki...
overexpression in Drosophila but not by YAP overexpression in mammalian cells [Dong et al. 2007]. We identified CTGF as a direct target gene of YAP-TEAD in mammalian cells. Interestingly, elevated CTGF levels have been detected in human cancers [Xie et al. 2001], and anti-CTGF antibody inhibited tumor growth and metastasis [Dornhofer et al. 2006]. This supports a possible role of CTGF in mediating the growth-stimulating and oncogenic function of YAP-TEAD. Although CTGF appears to play an important role in YAP-induced cell growth, it may not be required for YAP-induced EMT. This indicates that other genes may be involved in the biological function of YAP. Consistently, the TEAD-binding-defective YAP-S94A mutant can still induce expression of a fraction of the YAP-regulated genes. Furthermore, overexpression of the Sd-binding-defective Yki-S97A elicits a significantly reduced but still obvious overgrowth in Drosophila eyes and wings. These observations indicate that additional transcription factors may be used by YAP/Yki to regulate cell and tissue growth.

Materials and methods

Cell culture, transfection, and retroviral infection

HEK293 cells, HEK293-T cells, NIH-3T3 cells, and ACHN cells were cultured in DMEM [Invitrogen] containing 10% FBS [In-
vitrogen) and 50 µg/mL penicillin/streptomycin (P/S). MCF10A cells were cultured in DMEM/F12 [vitrogen] supplemented with 5% horse serum [vitrogen], 20 ng/mL EGF, 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, 100 ng/mL cholera toxin, and 50 µg/mL P/S. Transfection with lipofectamine was performed according to the manufacturer’s protocols [Agilent Technologies]. The hybridization intensity was extracted using the Agilent Feature Extraction Software. The bound probes were determined at a cut-off P-value of XDEV, which is a scaled log-ratio value generated from single-gene error model, <0.001.

Three-dimensional culture of MCF10A cells

The 3D culture of MCF10A cells was done as described [Debath et al. 2003]. Briefly, Growth Factor Reduced Matrigel was layered onto eight-well glass chamber slides to make a reconstituted basement membrane. MCF10A cells were seeded on top of that at a concentration of 5000 cells/well in assay medium containing 2% Matrigel and 5 ng/mL EGF. Cells were cultured in a 5% CO₂ humidified incubator at 37°C. The medium was replaced every 4 d.

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