INACTIVATION OF YAP-TEAD BY THE HIPPO PATHWAY IS INVOLVED IN GROWTH CONTROL AND CANCER

by

Bin Zhao

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
2009

Doctoral Committee:

Professor Kun-Liang Guan, Chair
Professor Randal J. Kaufman
Professor Benjamin L. Margolis
Associate Professor Roland P. Kwok
Assistant Professor Jiandie Lin

To my dearest parents who supported me all the way along

and to my beloved wife who is the best gift in my life.

ACKNOWLEDGEMENTS

I would first of all thank my mentor Dr. Kun-Liang Guan for his guidance, encouragement, and support during my graduate study. Dr. Guan not only gave me tons of help on my research, but also set me an example on how to be a scientist. His enthusiasm about science deeply affected me and firmed my confidence to pursue a scientific career.

I sincerely acknowledge the members of my thesis committee, Drs Randal J. Kaufman, Benjamin L. Margolis, Roland P. Kwok, and Jiandie Lin for their encouragement and guidance.

I would also acknowledge my collaborators for their excellent work including: Dr. Zhi-Chun Lai and his students Dr. Xiaomu Wei and Xin Ye for their work on all the YAP, Yki, and Scalloped transgenic flies; Dr. Georg Halder and his student Dr. Ryan S. Udan for their work on the Yki Dumbo mutant flies; Dr. Arul Chinnaiyan and his postdoc Dr. Jindan Yu for their work on ChIP-Chip and bioinformatics analysis; Dr. Joungmok Kim for his work on Lats2 kinase assay and NIH-3T3 cell transformation assay; Dr. Weiquan Li for generating many initial DNA constructs.

Many researchers provided me their reagents or expertise. I would also like to thank them here: Drs. Marius Sudol for pCMV-Flag-YAP2, pM-ErbB4-CTFΔK constructs, and the rabbit anti-YAP antibody; Tian Xu for Lats2; Brian Seed and Joseph Avruch for Mst2; Jiandie Lin for the Gal4-fusion transcription factors library and the 5×UAS-luciferase reporter; David M. Sabatini for the pLKO.1 vector; Sean Carroll for pGST-sd; Duojia Pan for pGal4-yki; Hongjiao Ouyang for RUNX2 and 6×OSE2-luc reporter; Dario R. Alessi for the PDK1 +/+ and -/- ES cell lysates; Ben Margolis for MCF10A cells; Keqiang Ye for the RT4-D6-P2T stable sells; Jing Yang for EMT marker

antibodies; Pan Zheng and Huiming zhang for assistance on IHC staining; and Taocong Jin for assistance on gene expression microarray.

The Guan lab members created a very supportive and comfortable environment for my research. I want to thank all of them. Especially, I would like to thank Dr. Ken Inoki for his hands-on training and constant encouragement; Tianqing Zhu for all her help in and out of the lab; Dr. Qian Yang for his friendship and all the fun he brought up.

Thanks also go to The University of Michigan Department of Biological Chemistry administrative staffs for their support, especially Beth Goodwin for her help on all kinds of issues.

Finally I would like to thank The University of Michigan Rackham Graduate School for the Predoctoral Fellowship and the Graduate Student Research Grant.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The mechanism of body and organ size control is an unsolved puzzle. Recent *Drosophila* genetics studies established the key role of the Hippo pathway and its downstream target Yki in organ size control. Yki is the homolog of the mammalian Yes-associated protein (YAP), a transcription co-activator. However, the regulation of YAP activity was not well understood. My study elucidated the mechanism of YAP regulation by the Hippo pathway in mammalian cells in response to cell density. At high cell density, phosphorylation of S127 by the Lats tumor suppressor kinase leads to cytoplasmic retention and inactivation of YAP. Attenuation of this phosphorylation of YAP or Yki potentiates their oncogenic transformation activity *in vitro* and growth-promoting function *in vivo*. YAP overexpression regulates gene expression in a manner opposite to cell density, and overcomes cell contact inhibition. Inhibition of YAP function restores contact inhibition in ACHN human cancer cell line. These evidence supports the involvement of Hippo-YAP pathway in cell contact inhibition.

As a transcription co-activator, YAP has to interact with transcription factors to activate gene expression. A critical transcription factor mediating YAP function was unknown. By screening a transcription factor library, I identified TEAD family transcription factors as the most potent YAP targets. Experiments further demonstrated that TEADs are required for YAP induced gene expression, cell growth, and oncogenic transformation. In addition, I identified CTGF (connective tissue growth factor) as a direct target gene of YAP-TEAD mediating their biological functions.

However, evidence suggests that YAP function also requires other transcription factors. WW domains of YAP, a structure mediating protein-protein interactions, are implicated in mediating interactions with other transcription factors. Consistently, I showed that the WW domains of YAP have 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.

The above data suggest a model that YAP plays a key role in the Hippo pathway to regulate cell proliferation, organ size, and oncogenic transformation by inducing expression of genes including CTGF through interaction with TEAD family and WW domain-binding transcription factors.

CHAPTER 1

INTRODUCTION

The Hippo pathway in *Drosophila*

In 1995, the first Hippo pathway component, *wts*, was uncovered by genetic mosaic screens in *Drosophila*. *wts* encodes a nuclear Dbf-2-related (NDR) family protein kinase (Justice et al. 1995; Xu et al. 1995). Mutation of *wts* leads to robust tissue overgrowth. Since 2002, similar screens have identified several other Hippo pathway components, including Salvador (Sav) (Kango-Singh et al. 2002; Tapon et al. 2002), Hippo (Hpo) (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003), and Mats(Lai et al. 2005). Together they form the core of the *Drosophila* Hippo pathway in which Hpo kinase, in association with an adaptor protein Sav, phosphorylates and activates Wts kinase, which is associated with an activating subunit Mats (Fig.1.1). Upstream of that might be Merlin (Mer) and Expanded (Ex), two ERM (ezrin/radixin/moesin) family cytoskeleton-related proteins (Hamaratoglu et al. 2006). Fat, a protocadherin might be further upstream (Bennett and Harvey 2006; Cho et al. 2006; Silva et al. 2006; Willecke et al. 2006; Tyler and Baker 2007). However, the biochemical mechanisms of Mer, Ex and Fat in regulation of the Hippo pathway core components are not clear.

The Hippo pathway limits organ size by inhibiting cell proliferation and promoting apoptosis (Edgar 2006). Such regulation is achieved at least in part by transcriptional activation of target genes like *cycE*, *diap1* (Edgar 2006) and *bantam* microRNA (Nolo et al. 2006; Thompson and Cohen 2006). Logically, the Hippo pathway should target some transcription regulators. Indeed, Yki, ortholog of the mammalian YAP,

a transcription co-activator, was identified as a Wts-interacting protein (Huang et al. 2005). Yki regulates transcription of the Hippo pathway target genes, and its overexpression phenocopies the loss of Hippo pathway components. Further biochemical studies showed that Wts directly phosphorylates Yki, which leads to Yki cytoplasmic retention and inactivation (Huang et al. 2005; Dong et al. 2007).

The discovery of Yki significantly advanced our understanding of the Hippo pathway. However, since Yki is a transcription co-activator, its promoter selectivity must be determined by its interacting transcription factors. It was recently reported that Scalloped (Sd), a critical regulator of proliferation and survival of wing imaginal disc cells (Halder et al. 1998; Simmonds et al. 1998), directly mediates Yki-induced gene expression and overgrowth phenotype (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008; Zhao et al. 2008). However, Sd is expressed in a narrower spectrum of cells while Yki and the Hippo pathway functions more ubiquitously (Campbell et al. 1992); *yki* mutant clones have more severe growth defects than *sd* mutant clones (Huang et al. 2005; Wu et al. 2008); and Sd-binding-defective Yki mutant elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (Zhao et al. 2008). Therefore, other transcription factors mediating the function of Yki and the Hippo pathway likely exist.

The Hippo pathway components in mammalian cells

Components of the Hippo pathway are highly conserved in mammals, including Mst1/2 (Hpo homolog), WW45 (also called Sav, Sav homolog), Lats1/2 (Wts homolog), Mob1 (Mats homolog), YAP and its paralog TAZ (both are Yki homologs), Mer (also called NF2, Mer homolog), and at a lesser degree FRMD6 (Ex homolog), and Fat4 (Fat homolog) (Fig.1.1). More strikingly, human YAP, Lats1, Mst2, and Mob1 can functionally rescue the corresponding *Drosophila* mutants *in vivo*, suggesting the functional conservation of these proteins in mammals (Edgar 2006). As Hpo in *Drosophila*, Mst plays a key role in the mammalian Hippo pathway as it phosphorylates all three other core components. Lats1/2 is phosphorylated by Mst1/2 on the activation

loop and hydrophobic motif, possibly with autophosphorylation involved (Chan et al. 2005). WW45 interacts with Mst through the SARAH domains in each other, and is then phosphorylated by Mst (Callus et al. 2006). Mob1 is also phosphorylated by Mst1/2, which enhances its interaction with Lats1 (Praskova et al. 2008). However, the mammalian Hippo pathway was not established until it was shown to inhibit YAP.

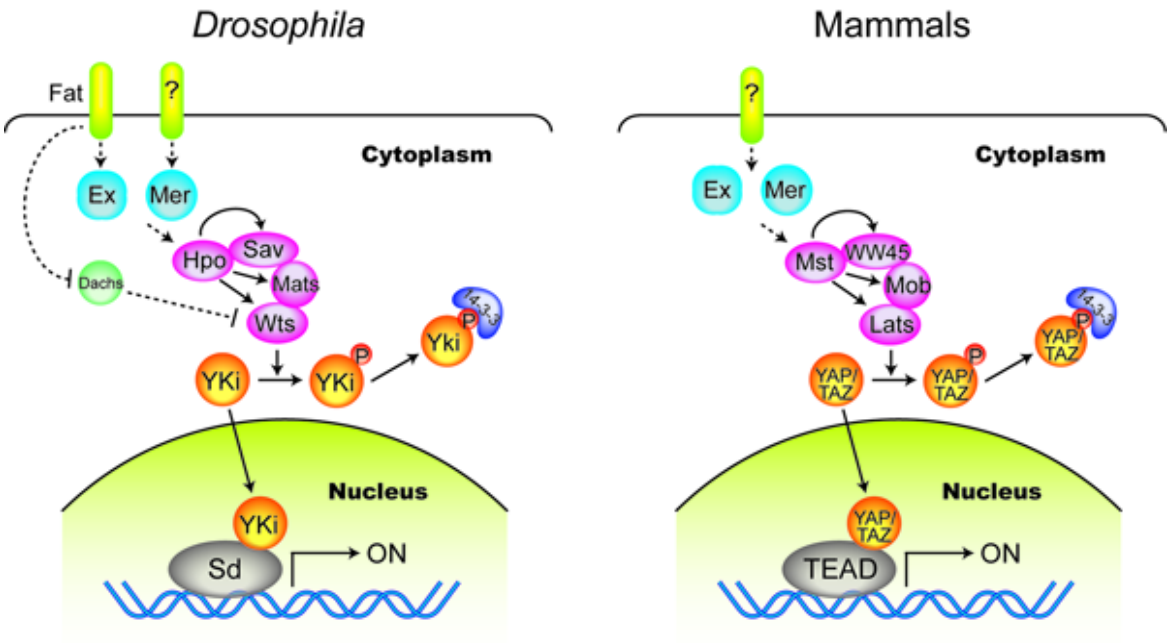


Figure 1.1 The Hippo pathway in *Drosophil*a and mammals.

Corresponding components in *Drosophila* and mammals are shown in the same color. The abbreviations used are as follows: Ex (Expanded), Mer (Merlin, also called NF2), Hpo (Hippo), Sav (Salvador), Mats (Mob as tumor suppressor), Wts (Warts), Yki (Yorkie), Sd (Scalloped), Mst (Mst1/2, also called STK4 and STK3, Hpo homolog), WW45 (Sav homolog), Mob (Mps One Binder kinase activator-like 1A/B, MOBKL1A/B, Mats homolog), Lats (Lats1/2, Wts homolog), YAP (Yes-associated protein, Yki homolog), TAZ (transcriptional co-activator with PDZ-binding motif, also called WWTR1, Yki homolog), and TEAD (TEA domain family member 1/2/3/4). Dashed arrows indicate unknown biochemical mechanism and question marks denote unknown components.

YAP is a transcription co-activator

YAP was first cloned as a protein bound to non-receptor tyrosine kinase c-Yes (Sudol 1994). It has several distinct domains as the human YAP2 shown in Fig.1.2. YAP also exists as YAP1, another splicing variant missing the second WW domain. Regulation of the switch between the two YAP isoforms is not clear. In general, YAP mRNA is ubiquitously expressed in a wide range of tissues, except peripheral blood leukocytes (Komuro et al. 2003). YAP is also expressed in the full developmental stages from blastocyst to perinatal (Morin-Kensicki et al. 2006).

However, the function of YAP remained enigmatic until it was shown to be a transcription co-activator (Yagi et al. 1999). YAP does not have any obvious DNA binding domain. However, when fused to Gal4 DNA binding domain, YAP could activate luciferase reporter as strong as VP16, a potent transcription activator. Therefore, it is categorized as a transcription co-activator. The transcription activation domain of YAP was further mapped to the C-terminal region. Interestingly, this region was found to be truncated in possibly dominant-negative YAP isoforms specifically expressed in neurons (Hoshino et al. 2006). YAP interacts with the PPXY motif of transcription factor PEBP2α (RUNX1 and RUNX2) mainly throught its first WW domain. Besides that, YAP has also been reported to co-activate other PPXY-motif-containing transcription factors, including ErbB4 cytoplasmic domain (Komuro et al. 2003) and p73 (Strano et al. 2001). YAP also binds to TEAD family transcription factors (Vassilev et al. 2001), which have four highly homologous proteins sharing a conserved DNA-binding TEA domain in human and mouse. The critical transcription factor mediating YAP function was unknown.

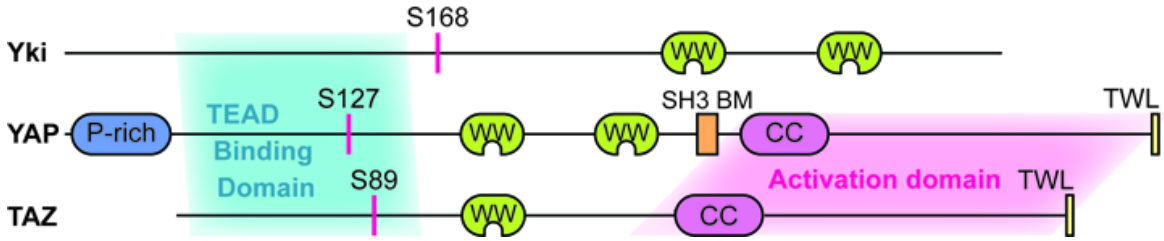


Figure 1.2 A schematic view of YAP, TAZ, and Yki.

YAP is a 65KDa protein with several distinct domains or motifs. It has a proline-rich (P-rich) region at the N-terminal, two tandem WW domains in the middle followed by a Src homology domain 3 binding motif (SH3 BM) PVKQPPPLAP, a coiled-coil domain (CC), and a C-terminal capped by TWL sequence, a PDZ domain ligand. The N-terminal (aa 47-154 in human YAP2, shaded in blue) of YAP was mapped to be the TEAD family transcription factors interaction domain [54], and the C-terminal of YAP (aa 292-488, shaded in pink) rich in serine, threonine, and acidic residues was shown to be a strong transcription activator [51]. The Lats phosphorylation and 14-3-3 binding critical S127 in human YAP2 and its equivalent in Yki and TAZ are also shown. TAZ is a mammalian paralog of YAP. The topology of Yki and TAZ are shown in similar fashion and the proteins are drawn in scale.

YAP as an oncoprotein

YAP is a potent growth promoter. Overexpression of YAP increases organ size in *Drosophila*. However, *yap* was termed a candidate oncogene only after it was shown to be in human chromosome 11q22 amplicon, which is detected in several human cancers (Overholtzer et al. 2006; Zender et al. 2006). Consistently, *yap* was shown to be amplified in human primary intracranial ependymomas by clinical study (Modena et al. 2006). 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 (Overholtzer et al. 2006); YAP cooperates with *myc* oncogene to stimulate tumor growth in nude mice (Zender et al. 2006); and more interestingly, transgenic mice with liver-specific YAP overexpression show a dramatic increase in liver size and eventually develop tumors (Camargo et al. 2007; Dong et al. 2007). The above evidence strongly indicates the function of *yap* as an oncogene.

The oncogenic function of YAP is further supported by the tumor suppressor function of its inhibitory upstream Hippo pathway components. Lats1 knockout leads to soft-tissue sarcoma and ovarian tumor development (St John et al. 1999). *mob*, an activating subunit of Lats, is mutated in both human and mouse cancer cells (Lai et al. 2005). Loss-of-function mutation of WW45 has been observed in several human cancer cell lines (Tapon et al. 2002). Furthermore, a recent report showed that knockout of *ww45* leads to hyperplasia and differentiation defects in mouse embryonic epithelial structures (Lee et al. 2008). Mer, which is further upstream of the Hippo pathway, is a well-established human tumor suppressor (Evans et al. 2000). Therefore, the Hippo pathway consists of many proven or candidate tumor suppressors that inhibit YAP oncoprotein.

Here I present data suggesting a model that YAP plays a key role in the Hippo pathway to regulate cell proliferation, organ size, and oncogenic transformation by inducing expression of genes including CTGF through interaction with TEAD family and WW domain-binding transcription factors.

Bibliography

Bennett, F.C. and Harvey, K.F. 2006. Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. *Curr Biol* **16**(21): 2101-2110.

Callus, B.A., Verhagen, A.M., and Vaux, D.L. 2006. Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. *FEBS J* **273**(18): 4264-4276.

Camargo, F.D., Gokhale, S., Johnnidis, J.B., Fu, D., Bell, G.W., Jaenisch, R., and Brummelkamp, T.R. 2007. YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr Biol* **17**(23): 2054-2060.

Campbell, S., Inamdar, M., Rodrigues, V., Raghavan, V., Palazzolo, M., and Chovnick, A. 1992. The scalloped gene encodes a novel, evolutionarily conserved transcription factor required for sensory organ differentiation in Drosophila. *Genes Dev* **6**(3): 367-379.

Chan, E.H., Nousiainen, M., Chalamalasetty, R.B., Schafer, A., Nigg, E.A., and Sillje, H.H. 2005. The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene* **24**(12): 2076-2086.

Cho, E., Feng, Y., Rauskolb, C., Maitra, S., Fehon, R., and Irvine, K.D. 2006. Delineation of a Fat tumor suppressor pathway. *Nat Genet* **38**(10): 1142-1150.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S.A., Gayyed, M.F., Anders, R.A., Maitra, A., and Pan, D. 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* **130**(6): 1120-1133.

Edgar, B.A. 2006. From cell structure to transcription: Hippo forges a new path. *Cell* **124**(2): 267-273.

Evans, D.G., Sainio, M., and Baser, M.E. 2000. Neurofibromatosis type 2. *J Med Genet* **37**(12): 897-904.

Goulev, Y., Fauny, J.D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. 2008. SCALLOPED Interacts with YORKIE, the Nuclear Effector of the Hippo Tumor-Suppressor Pathway in Drosophila. *Curr Biol* **18**(6): 435-441.

Halder, G., Polaczyk, P., Kraus, M.E., Hudson, A., Kim, J., Laughon, A., and Carroll, S. 1998. The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in Drosophila. *Genes Dev* **12**(24): 3900-3909.

Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., and Halder, G. 2006. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. *Nat Cell Biol* **8**(1): 27-36.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. 2003. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**(4): 457-467.

Hoshino, M., Qi, M.L., Yoshimura, N., Miyashita, T., Tagawa, K., Wada, Y., Enokido, Y., Marubuchi, S., Harjes, P., Arai, N., Oyanagi, K., Blandino, G., Sudol, M., Rich, T., Kanazawa, I., Wanker, E.E., Saitoe, M., and Okazawa, H. 2006. Transcriptional repression induces a slowly progressive atypical neuronal death associated with changes of YAP isoforms and p73. *J Cell Biol* **172**(4): 589-604.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**(3): 421-434.

Jia, J., Zhang, W., Wang, B., Trinko, R., and Jiang, J. 2003. The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. *Genes Dev* **17**(20): 2514-2519.

Justice, R.W., Zilian, O., Woods, D.F., Noll, M., and Bryant, P.J. 1995. The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes Dev* **9**(5): 534-546.

Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P.R., Bellen, H.J., and Halder, G. 2002. Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. *Development* **129**(24): 5719-5730.

Komuro, A., Nagai, M., Navin, N.E., and Sudol, M. 2003. WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. *J Biol Chem* **278**(35): 33334-33341.

Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., and Li, Y. 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120**(5): 675-685.

Lee, J.H., Kim, T.S., Yang, T.H., Koo, B.K., Oh, S.P., Lee, K.P., Oh, H.J., Lee, S.H., Kong, Y.Y., Kim, J.M., and Lim, D.S. 2008. A crucial role of WW45 in developing epithelial tissues in the mouse. *EMBO J* **27**(8): 1231-1242.

Modena, P., Lualdi, E., Facchinetti, F., Veltman, J., Reid, J.F., Minardi, S., Janssen, I., Giangaspero, F., Forni, M., Finocchiaro, G., Genitori, L., Giordano, F., Riccardi, R., Schoenmakers, E.F., Massimino, M., and Sozzi, G. 2006. Identification of tumor-specific molecular signatures in intracranial ependymoma and association with clinical characteristics. *J Clin Oncol* **24**(33): 5223-5233.

Morin-Kensicki, E.M., Boone, B.N., Howell, M., Stonebraker, J.R., Teed, J., Alb, J.G., Magnuson, T.R., O'Neal, W., and Milgram, S.L. 2006. Defects in yolk sac

vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65. *Mol Cell Biol* **26**(1): 77-87.

Nolo, R., Morrison, C.M., Tao, C., Zhang, X., and Halder, G. 2006. The bantam microRNA is a target of the hippo tumor-suppressor pathway. In *Curr Biol*, pp. 1895-1904.

Overholtzer, M., Zhang, J., Smolen, G.A., Muir, B., Li, W., Sgroi, D.C., Deng, C.X., Brugge, J.S., and Haber, D.A. 2006. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc Natl Acad Sci U S A* **103**(33): 12405-12410.

Pantalacci, S., Tapon, N., and Leopold, P. 2003. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat Cell Biol* **5**(10): 921-927.

Praskova, M., Xia, F., and Avruch, J. 2008. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. *Curr Biol* **18**(5): 311-321.

Silva, E., Tsatskis, Y., Gardano, L., Tapon, N., and McNeill, H. 2006. The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. *Curr Biol* **16**(21): 2081-2089.

Simmonds, A.J., Liu, X., Soanes, K.H., Krause, H.M., Irvine, K.D., and Bell, J.B. 1998. Molecular interactions between Vestigial and Scalloped promote wing formation in Drosophila. *Genes Dev* **12**(24): 3815-3820.

St John, M.A., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M.L., Brownstein, D.G., Parlow, A.F., McGrath, J., and Xu, T. 1999. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat Genet* **21**(2): 182-186.

Strano, S., Munarriz, E., Rossi, M., Castagnoli, L., Shaul, Y., Sacchi, A., Oren, M., Sudol, M., Cesareni, G., and Blandino, G. 2001. Physical interaction with Yes-associated protein enhances p73 transcriptional activity. *J Biol Chem* **276**(18): 15164-15173.

Sudol, M. 1994. Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. *Oncogene* **9**(8): 2145-2152.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. 2002. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**(4): 467-478.

Thompson, B.J. and Cohen, S.M. 2006. The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. *Cell* **126**(4): 767-774.

Tyler, D.M. and Baker, N.E. 2007. Expanded and fat regulate growth and differentiation in the Drosophila eye through multiple signaling pathways. *Dev Biol* **305**(1): 187-201.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat Cell Biol* **5**(10): 914-920.

Vassilev, A., Kaneko, K.J., Shu, H., Zhao, Y., and DePamphilis, M.L. 2001. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev* **15**(10): 1229-1241.

Willecke, M., Hamaratoglu, F., Kango-Singh, M., Udan, R., Chen, C.L., Tao, C., Zhang, X., and Halder, G. 2006. The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Curr Biol* **16**(21): 2090-2100.

Wu, S., Huang, J., Dong, J., and Pan, D. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**(4): 445-456.

Wu, S., Liu, Y., Zheng, Y., Dong, J., and Pan, D. 2008. The TEAD/TEF Family Protein Scalloped Mediates Transcriptional Output of the Hippo Growth-Regulatory Pathway. *Dev Cell*.

Xu, T., Wang, W., Zhang, S., Stewart, R.A., and Yu, W. 1995. Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. *Development* **121**(4): 1053-1063.

Yagi, R., Chen, L.F., Shigesada, K., Murakami, Y., and Ito, Y. 1999. A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. *Embo J* **18**(9): 2551-2562.

Zender, L., Spector, M.S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S.T., Luk, J.M., Wigler, M., Hannon, G.J., Mu, D., Lucito, R., Powers, S., and Lowe, S.W. 2006. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* **125**(7): 1253-1267.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. 2008. The TEAD/TEF Family of Transcription Factor Scalloped Mediates Hippo Signaling in Organ Size Control. *Dev Cell*.

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Lin, J.D., Wang, C.Y., Chinnaiyan, A.M., Lai, Z.C., and Guan, K.L. 2008. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* **22**(14): 1962-1971.

CHAPTER 2

INACTIVATION OF YAP ONCOPROTEIN BY THE HIPPO PATHWAY IS INVOLVED IN CELL CONTACT INHIBITION AND TISSUE GROWTH CONTROL

Abstract

The Hippo pathway plays a key role in organ size control by regulating cell proliferation and apoptosis in *Drosophila*. Although recent genetic studies have shown that the Hippo pathway is regulated by the NF2 and Fat tumor suppressors, the physiological regulation of this pathway is unknown. Here we show that in mammalian cells, the transcription co-activator YAP (Yes-associated protein) is inhibited by cell density via the Hippo pathway. Phosphorylation by the Lats tumor suppressor kinase leads to cytoplasmic retention and inactivation of the YAP oncoprotein. Furthermore, attenuation of this phosphorylation of YAP or Yki, the *Drosophila* homolog of YAP, potentiates their growth-promoting function *in vivo*. Moreover, YAP overexpression regulates gene expression in a manner opposite to cell density, and is able to overcome cell contact inhibition. Inhibition of YAP function restores contact inhibition in a human cancer cell line bearing deletion of Sav, a Hippo pathway component. Interestingly, we observed that YAP protein is elevated and nuclear localized in some human liver and prostate cancers. Our observations demonstrate that YAP plays a key role in the Hippo pathway to control cell proliferation in response to cell contact.

Introduction

Body and organ size of metazoans is determined by cell number and cell size. The opposing action of cell proliferation and apoptosis controls cell number in particular tissue and organs (Conlon and Raff 1999). Recent genetic studies in *Drosophila* have shown that the Hippo signaling pathway plays a key role in restricting organ size by controlling both cell proliferation and apoptosis (Edgar 2006; Harvey and Tapon 2007; Pan 2007). Hippo (Hpo) is a Ste20 family protein kinase which complexes with a regulatory scaffold protein Salvador (Sav) (Kango-Singh et al. 2002; Tapon et al. 2002; Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003). The Hpo/Sav complex phosphorylates and activates Warts (Wts), a NDR family protein kinase. Wts has an activating subunit Mats (Mob as tumor suppressor) (Lai et al. 2005; Wei et al. 2007). The Wts/Mats complex inhibits Yorkie (Yki), a transcription co-activator (Huang et al. 2005), possibly via direct phosphorylation, although the precise mechanism has yet to be determined. In *Drosophila*, key downstream targets of Yki include *cyclin E, diap-1*, and the *bantam* micro RNA (Huang et al. 2005; Nolo et al. 2006; Thompson and Cohen 2006).

Although elusive for several years, the signals upstream of Hpo are now emerging. The NF2 tumor suppressor, also known as Merlin (Mer), and Expanded (Ex), two ezrin/radixin/moesin (ERM) family actin binding proteins (McClatchey and Giovannini 2005; Okada et al. 2007), have been shown to positively regulate the Hippo pathway in *Drosophila* (Hamaratoglu et al. 2006). Interestingly, genetic data indicate that Fat, a protocadherin tumor suppressor, also functions upstream of Hpo (Bennett and Harvey 2006; Cho et al. 2006; Hariharan 2006; Silva et al. 2006; Willecke et al. 2006; Tyler and Baker 2007; Yin and Pan 2007). The fact that Fat may interact with another protocadherin, Dachsous, at the cell surface (Matakatsu and Blair 2004; Halbleib and Nelson 2006) suggests an exciting possibility that the Hippo pathway may be involved in cell growth regulation in response to cell-cell contact.

Components of the Hippo pathway are highly conserved in mammals, including YAP, Lats1/2, Mob, Mst1/2, Sav, Merlin, Ex1/2, and Fat4 (Yki, Wts, Mats, Hpo, Sav, Mer, Expanded, and Fat homologs respectively). Human YAP, Lats1, Mst2, and Mob1

can functionally rescue the respective *Drosophila* mutants, suggesting the functional conservation of these proteins in mammals (Edgar 2006). Interestingly, YAP has recently been shown to be a candidate oncogene in the human chromosome 11q22 amplicon (Overholtzer et al. 2006; Zender et al. 2006). In addition, mutations of Lats1/2, Sav, and Mob have been implicated in tumorigenesis (St John et al. 1999; Tapon et al. 2002; Lai et al. 2005; Takahashi et al. 2005; Harvey and Tapon 2007). In spite of their conservation and intimate relationship with cancer, the Hippo pathway has not been systematically studied in mammalian cells.

A fundamental property of a normal cell is to cease proliferation upon reaching confluence, a phenomenon referred to as cell contact inhibition (Eagle and Levine 1967). In contrast, cancer cells are able to escape cell contact inhibition, which enhances their ability to invade host tissues and metastasize (Hanahan and Weinberg 2000). This is also one of the most commonly used criteria for cellular transformation *in vitro* (Abercrombie 1979). Although activation of oncogenes and inactivation of tumor suppressor genes can prevent contact inhibition, the precise molecular mechanism is not clear.

In this report we show that YAP is regulated by the Hippo pathway and may play an important role in mediating cell contact inhibition. YAP is phosphorylated and inhibited by the Lats tumor suppressor, and this phosphorylation results in its association with 14-3-3 and cytoplasmic localization. This regulatory mechanism is utilized in YAP regulation by cell density and is likely conserved in *Drosophila*. Furthermore, overexpression of YAP antagonizes density-dependent gene regulation and contact inhibition, whereas expression of dominant-negative YAP restores contact inhibition in a human cancer cell line bearing a deletion of Sav. Moreover, we showed that YAP expression levels and nuclear localization are strongly elevated in some human cancers.

Materials and Methods

Antibodies, Plasmids, and Materials

Anti-YAP, anti-Lats, anti-NF2, anti-14-3-3 theta, and anti-Actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-YAP (S127), anti-Akt, anti-phospho-Akt (S473), anti-phospho-Akt (T308), and anti-phospho-GSK3 α/β (S21/9) were obtained from Cell Signaling (Beverly, MA). Anti-Lats2 antibody was obtained from Bethyl Laboratories (Montgomery, TX). Anti-α-Tubulin and anti-Flag antibodies were obtained from Sigma (St. Louis, MO). Anti-HA and Anti-Myc antibodies were obtained from Covance (Philadelphia, PA). Alexa Fluor 488 mouse anti-human Ki67 was obtained from BD Biosciences (San Jose, CA). Anti-GSK3 antibody was obtained from Upstate (Charlottesville, VA). Anti-GST antibody was obtained from EMD Biosciences (Madison, WI). Alexa Fluor 488 or 594 conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Horseradish peroxidase conjugated secondary antibodies were obtained from Amersham (Buckinghamshire, UK).

The pCMV-Flag-YAP2 construct was kindly provided by Dr. Marius Sudol, and YAP2 was subcloned into the pGEX GST-fusion vector, the pQCXIH-Myc retrovirus vector, and the pUAST vector. pcDNA3-HA-Lats2 was a gift from Dr. Tian Xu. Flag-Mst2 was kindly provided by Dr. Brian Seed and Dr. Joseph Avruch and was subcloned into a pcDNA3-HA vector. Merlin, Expanded, Salvador, and Mob were cloned from human brain or HeLa cDNA libraries into the pcDNA3-HA vector. TEAD4 was cloned from a HeLa cDNA library into the PRK5-Myc vector. The 5×UAS-luciferase reporter and the Gal4-TEAD4 and Gal4-Foxo3 constructs were generously provided by Dr. Jiandie Lin. The Myc-14-3-3β and Akt constructs have been described (Inoki et al., 2002; Li et al., 2002). The YAP2 S61A, S109A, S127A, S163/164A, S381A, and 5SA mutants, and the Mst2 and Lats2 KR mutants were generated by site-directed mutagenesis. The YAP2-5SA-ΔC construct was generated by truncating the C-terminus of YAP2-5SA mutant from amino acid Q291. PDK1 +/+ and -/- ES cell lysates were kindly provided by Dr. Dario R. Alessi (University of Dundee, UK). LY294002 and wortmannin were purchased from Calbiochem (La Jolla, CA). Insulin was from Invitrogen (Carlsbad, CA).

Cell culture, Transfection, and Retroviral Infection

HEK293 cells, 293T cells, HeLa cells, NIH-3T3 cells, MEF cells, ACHN cells and the RT4-D6-P2T Schwannoma cells were cultured in DMEM (Invitrogen) containing 10% FBS (Invitrogen) and 50μg/ml penicillin/streptomycin (P/S). MCF10A cells were cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20ng/ml EGF, 0.5μg/ml hydrocortisone, 10μg/ml insulin, 100ng/ml cholera toxin, and 50μg/ml P/S. Transfection with lipofectamine was performed according to the manufacturer's instructions.

To generate wild type or mutant YAP2 expressing cells, retrovirus infection was performed by transfecting 293 Phoenix retrovirus packaging cells with empty vector or pQCXIH-YAP2 constructs. 48 hours after transfection, retroviral supernatant was supplemented with 5μg/ml polybrene, filtered through a 0.45μm filter, and used to infect MCF10A, NIH-3T3, or ACHN cells. 36 hours after infection, cells were selected with 200μg/ml hygromycin (Roche) in culture medium. The RT4-D6-P2T Schwannoma cells with inducible Merlin expression have been described before (Morrison et al. 2001; Rong et al. 2004).

Immunofluorescence Staining

For immunofluorescence staining, cells were cultured on cover slips to appropriate density. Cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton X-100. After blocking in 3% BSA for 30 min, slides were incubated with first antibody diluted in 1% BSA for 1.5 hours. After washing with PBS, slides were incubated with Alexa Fluor 488 or 594 conjugated secondary antibodies (1:1000 dilution) for 1.5 hours. The slides were then washed and mounted.

Immunoprecipitation and Kinase Assay

For the Lats2 and Mst2 kinase assays, HEK293 cells were transfected with HA-Lats2 or Flag-Mst2. 48 hours post-transfection, cells were lysed with lysis buffer [50mM HEPES (pH 7.5), 150mM NaCl, 1mM EDTA, 1% NP-40, 10mM pyrophosphoate, 10mM glycerophosphate, 50mM NaF, 1.5mM Na_3VO_4, protease inhibitor cocktail (Roche), 1mM DTT, 1mM PMSF], and immunoprecipitated with anti-HA or anti-Flag antibodies. The immunoprecipitates were washed 3 times with lysis buffer, followed by once with wash buffer [40mM HEPES, 200mM NaCl] and once with kinase assay buffer [30mM HEPES, 50mM potassium acetate, 5mM $MgCl_2$]. The immunoprecipitated Lats2 or Mst2 was subjected to a kinase assay in the presence of 500μM cold ATP, 10μCi [γ-^{32}P]ATP, and 1μg GST-YAP2 expressed and purified from *E.coli* as substrate. The reaction mixtures were incubated at 30°C for 30 min, terminated with SDS sample buffer, and subjected to SDS-PAGE and autoradiography. The same procedure was used for endogenous Lats2 kinase assay except that endogenous Lats2 immunoprecipitated from NIH-3T3 cells was used.

Luciferase Assay

For the luciferase reporter assay, 293T cells were seeded in 12 well plates. 5×UAS-luciferase reporter, CMV-β-gal, and indicated plasmids were co-transfected as described previously. 36 hours after transfection, cells were lysed and luciferase activity was assayed using the enhanced luciferase assay kit obtained from BD Biosciences (San Jose, CA) following the manufacturer's instructions. All luciferase activities were normalized to β-galactosidase activity.

BrdU Labeling and Flow Cytometric Analysis

For cell cycle progression analysis, cells were cultured to desired confluence. Cells were then labeled with 5-bromo-2′-deoxyuridine (BrdU) and analyzed by flow cytometry using the FITC BrdU Flow Kit obtained from BD Biosciences (San Jose, CA)

following the manufacturer's instructions. Briefly, cells were pulse labeled with 10 μM BrdU in culture medium for 30 min. After trypsinization and PBS wash, cells were fixed and permeabilized. Incorporated BrdU was exposed by DNase treatment and then stained by FITC-conjugated anti-BrdU antibody. Total DNA was stained by 7-AAD (7-amino-actinomycin D). Data was collected on a BD FACSCalibur and analyzed with CellQuest Pro software.

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 Cybergreen (Applied Biosystems). Relative abundance of mRNA was calculated by normalization to hypoxanthine phosphoribosyltransferase 1 (HPRT) mRNA.

Gene Expression Microarray Analysis

For analysis of gene expression in YAP overexpression cells, the cells were cultured to 90% confluency before harvest. For comparing gene expression in low and high density cultures, cells were seeded at different densities and harvested at 30% or complete confluency. Total RNA was extracted with Trizol (Invitrogen) followed by further purification using the RNeasy kit (Qiagen). Biotinylated cRNAs were then prepared according to the Affymetrix standard labeling protocol. The biotinylated cRNAs were then fragmented and hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 Array or the Human Genome U133 Plus 2.0 Array, respectively. Chips were washed and stained with Streptavidin R-phycoerythrin (Invitrogen). After scanning the chips, the data were analyzed using GCOS software. Scaling was performed with a target intensity of 500 to facilitate the comparison of multiple arrays. A cut-off value of 0.05 was applied to the detection *p* value to assign a present (P), marginal (M), or absent (A) call to each

probe set. A signal value was calculated using the One-Step Tukey's Biweight Estimate to represent the relative abundance of a transcript. Up or down regulation of a gene is determined by two criteria: first, at least one P call in the two samples being compared; second, at least two fold change (or indicated) of the signal value. The microarray analysis was done at the Molecular Biology Core Laboratory (University of Michigan, School of Dentistry).

RNA Interference

Smart pool short interfering RNA (siRNA) oligonucleotides toward human Lats1 or Lats2 and control siRNA toward firefly luciferase were purchased from Dharmacon (Denver, CO). siRNAs were transfected into HeLa or HEK293 cells twice with a 24 hours interval. Cell lysate was made 48 hours post-transfection.

Drosophila Genetics

For *in vivo* functional analysis of YAP2/Yki, full-length cDNAs of *YAP2* or *yki* were cloned into a transformation vector *pUAST*. Multiple transgenic fly lines were generated for each of the following DNA constructs: *pUAS-Flag-YAP2* (30 lines), *pUAS-Flag-YAP2*^{S127A} (7 lines), and *pUAS-yki-V5* (9 lines). *GMR-Gal4* drives eye-specific expression of *UAS* transgenes. To measure β-galactosidase activity, third instar larval eye discs were fixed in 1% glutaraldehyde for 15 minutes at room temperature, and the staining reaction was carried out at 37°C for 20 hours. Scanning electron microscopy (SEM) was used to reveal adult eye phenotypes.

Mutagenesis screen: *yw* males were fed a 1% sucrose solution containing 15 mM of ethymethanesulfonate (EMS [ICN Biomedicals]) over a 16 hour period of time. Males were then allowed to recover for 24 hours on standard corn meal-based fly food before they were crossed to *nubbin-Gal4 UAS-hpo*$^{\Delta Inh2a}$ (*NH*) females. *NH* flies show a small wing phenotype caused by the overexpression of Hpo in the developing wing pouch.

Single F1 flies displaying enhanced or suppressed wing phenotypes were selected and backcrossed to *NH* flies. Flies with transmitted modifier mutations were crossed to balancer stocks to the isogenize modifier chromosomes on the X, 2nd, and 3rd chromosomes.

Generation of mutant clones: Mutant clones were induced by using the FLP/FRT system(Newsome et al., 2000; Xu and Rubin, 1993). *yki* mutant clones in eye imaginal discs were generated by flipping yki^{Dbo} alleles against ubiGFP-marked FRT42D chromosomes. For adult clones, yki^{Dbo} alleles were flipped against an FRT42D cell-lethal P[w+] chromosome in a w^- background.

Immunohistochemistry: Antibody staining of imaginal discs was done as described (Kango-Singh et al., 2002). The following antibodies were used (source and dilutions in parentheses): mouse α-Dlg (DSHB, 1:300), rabbit α-Ex (A. Laughon, 1:1500), mouse α-BrdU (Becton-Dickinson, 1:50), and mouse α-CycE (H. Richardson, 1:40). Secondary antibodies were donkey Fab fragments from Jackson ImmunoResearch. BrdU incorporation was done as described by incorporating BrdU for 1 hr.

Results

YAP Localization and Phosphorylation Are Regulated by Cell Density

YAP is a transcription co-activator and a candidate oncogene, but neither its function in cancers nor its physiological regulation has been established. Interestingly, we found that YAP localization was regulated by cell density (Fig.2.1A). At low density, YAP was predominantly localized in the nuclei of NIH-3T3 cells. In contrast, YAP translocated to the cytoplasm at high density. Similar observations were made in the MCF10A human breast epithelial cell line (Fig.2.1A). This translocation was unlikely due to differential medium conditions because in cell colonies, YAP was preferentially localized to nuclei in cells at the edge but displayed cytoplasmic localization in cells towards the center (Fig.2.1B). Given the fact that YAP is a transcription co-activator

acting in the cell nucleus (Yagi et al. 1999), our results indicate that YAP may be inhibited by high cell density.

Besides translocation, YAP from high-density cultures displayed a slower electrophoretic migration (Fig.2.1C). This density dependent mobility shift was due to phosphorylation because phosphatase treatment converted YAP to the fast migrating form, suggesting that YAP phosphorylation is regulated by cell density. Together, the above observations indicate a possible relationship between YAP phosphorylation and cytoplasmic localization upon high cell density.

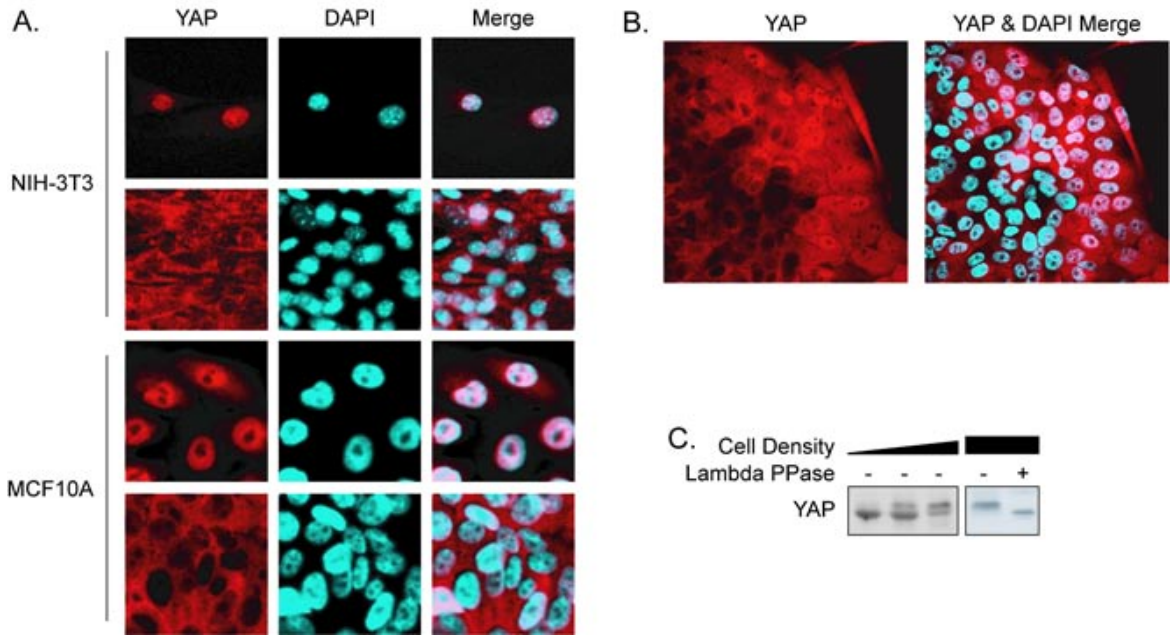


Figure 2.1 YAP localization and phosphorylation are regulated by cell density.

A. YAP localization is affected by cell density. NIH-3T3 and MCF10A cells were cultured sparsely or to confluence. YAP was stained with anti-YAP antibody.

B. MCF10A cells at the edge of a large colony have high nuclear YAP. YAP was stained with anti-YAP antibody.

C. High cell density induces YAP phosphorylation. NIH-3T3 cell lysates from cells at different densities were probed with anti-YAP antibody. Lambda phosphatase treatment is indicated.

The Hippo Pathway Regulates YAP Phosphorylation, Activity, and Localization

In *Drosophila*, it has been reported that Yki, the YAP homolog, is inhibited by the Hippo pathway, possibly via phosphorylation (Huang et al. 2005). Therefore, we tested effects of the Hippo pathway on YAP phosphorylation. All cDNAs used in the cell culture studies are of human or mouse origin. YAP2, one of the two alternatively spliced forms of human *yap*, was co-expressed with the Hippo pathway kinases Mst2 or Lats2. We found that expression of Mst2 or Lats2 caused a modest mobility shift of YAP2 that was further enhanced by Sav and Mob, the respective regulatory subunits of Mst2 and Lats2 (Fig.2.2A). Moreover, co-expression of both Mst2 and Lats2 resulted in a dramatic mobility shift of YAP2. These results indicate that ectopic expression of Mst2 and Lats2 induces YAP2 phosphorylation.

In order to test the possibility of direct phosphorylation of YAP2 by Lats2, we performed an *in vitro* kinase assay using purified GST-YAP2 and immunoprecipitated Lats2. As shown in Fig.2.2B, Lats2 but not the kinase-inactive Lats2-KR, phosphorylated YAP2. In contrast, Mst2 poorly phosphorylated GST-YAP2, even though it had much stronger autophosphorylation than that of Lats2 (Fig.2.3A). These data demonstrate that Lats2 directly phosphorylates YAP2, while Mst2 stimulates YAP2 phosphorylation indirectly *in vivo*, perhaps by activating Lats2.

YAP has been shown to interact with and activate the TEAD family transcription factors, which have four highly conserved members (Vassilev et al. 2001). To assess the effect of phosphorylation on YAP activity, we utilized a reporter system consisting of a 5×UAS-luciferase reporter and a Gal4 DNA binding domain fused to TEAD4 (Gal4-TEAD4). In the absence of YAP, Gal4-TEAD4 had low basal activity. However, when YAP2 was co-transfected, the reporter was strongly activated (Fig.2.2C). Co-expression of Lats2 or Mst2 but not the kinase-inactive mutants resulted in a dose-dependent inhibition of the reporter (Fig.2.3B, 2.3C). Reminiscent of the effect seen on phosphorylation, YAP2 activity was further inhibited by co-expressing Mst2/Sav or Lats2/Mob, and even more dramatically inhibited by a combination of all four proteins (Fig.2.2C). This inhibition of YAP2 activity was also observed in COS7 and HeLa cells

(data not shown). We also tested the effect of Merlin and Expanded on YAP2 activity. Co-expression of either caused a modest but reproducible inhibition of YAP2 activity (Fig.2.2D). Furthermore, Merlin and Expanded enhanced the inhibition of YAP2 by Mst2 and Lats2. Consistently, Merlin also caused a mobility shift of YAP2 (Fig.2.3D).

Next, we addressed whether Mst2 and Lats2 affected YAP localization. In HeLa cells, endogenous YAP was localized in the nucleus at low cell density (Fig.2.2E). However, expression of Lats2 but not the kinase-inactive mutant caused a dramatic redistribution of YAP to the cytoplasm (Fig.2.2E). Similarly, expression of Mst2 but not the kinase-inactive mutant increased cytoplasmic YAP, although less dramatically. Expression of Merlin also resulted in YAP cytoplasmic translocation, supporting the role of Merlin in the Hippo pathway in mammalian cells. These results suggest that activation of the Hippo pathway may cause cytoplasmic translocation of YAP through phosphorylation by Lats.

Schwannoma is the major tumor type associated with Merlin mutation. We examined YAP localization in the RT4-D6-P2T rat Schwannoma cell line, which is incapable of inducing Merlin expression at high cell density as what normal Schwann cells do (Morrison et al. 2001). We observed that the majority of Schwannoma cells showed nuclear YAP localization even under high density (Fig.2.2F). Interestingly, expression of Merlin wild type, but not a cancer derived L64P mutant, restored YAP cytoplasmic translocation. Together, these results further support the involvement of Merlin and the Hippo pathway in the regulation of YAP translocation in response to cell density.

Figure 2.2 The Hippo pathway regulates YAP phosphorylation, activity, and localization.

A. Co-expression of Mst2 and Lats2 decreases YAP2 mobility. Flag-YAP2 was co-transfected with indicated plasmids into HEK293 cells. Western blot was performed as indicated.

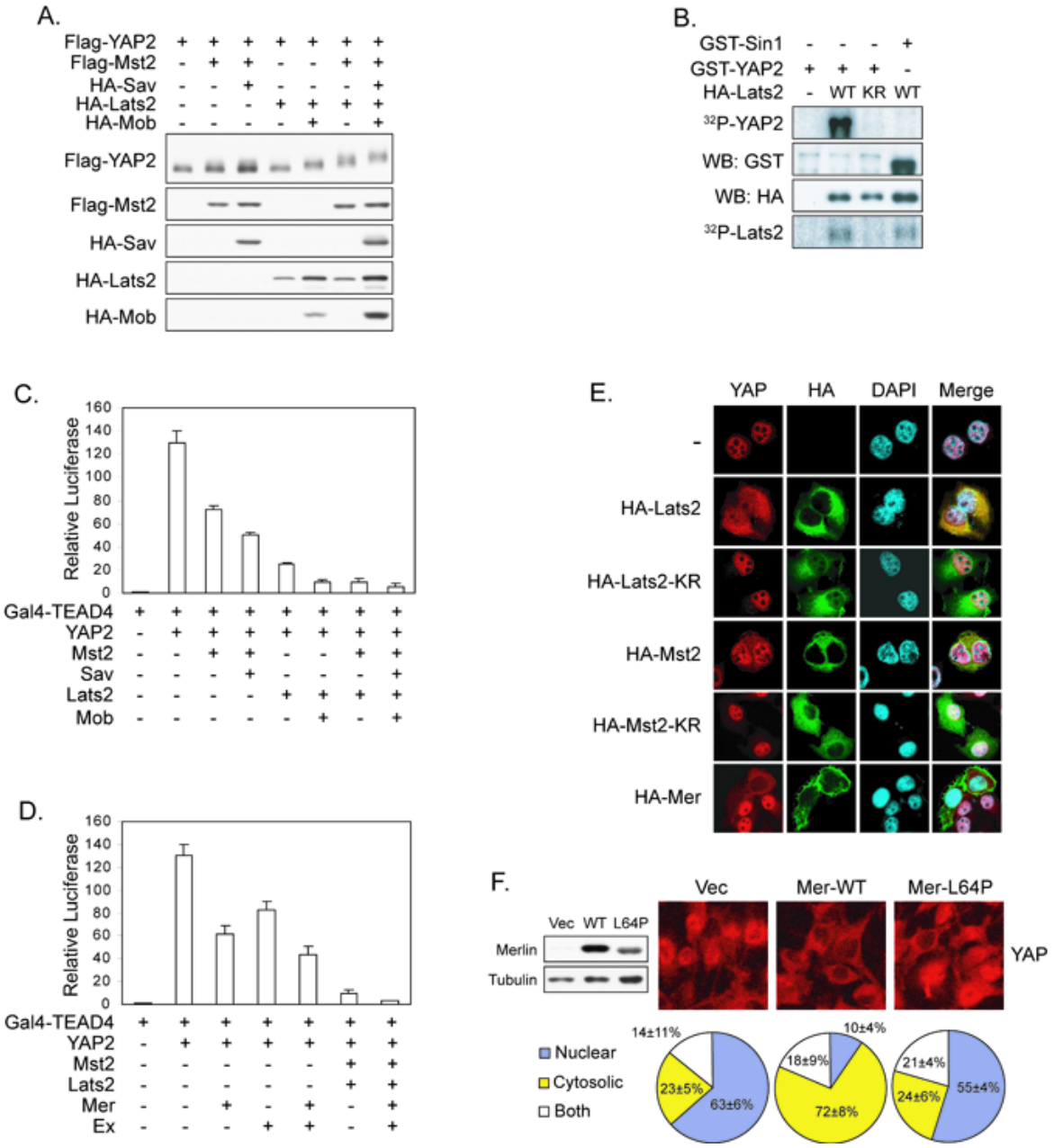
B. *In vitro* phosphorylation of YAP2 by Lats2. HA-Lats2 was immunoprecipitated from transfected HEK293 cells. *In vitro* kinase assay was performed using purified GST-YAP2 as a substrate in the presence of ^{32}P-ATP. GST-Sin1 was used as a negative control. KR denotes kinase-inactive mutant.

C. YAP2 activity is inhibited by Mst2 and Lats2. Indicated plasmids were co-transfected with a 5×UAS-luciferase reporter and a CMV-β-gal construct into 293T cells. Luciferase activity was measured and normalized to β-galactosidase activity. The Ex used is human FRMD6.

D. YAP2 activity is inhibited by Merlin and Expanded. Experiments are similar as in panel C.

E. Activation of the Hippo pathway causes YAP cytoplasmic localization. HeLa cells were transfected with indicated plasmids. Endogenous YAP2 was stained to visualize the localization.

F. Cell density induced YAP translocation is Merlin dependent. RT4-D6-P2T Schwannoma cell lines with empty vector, inducible wild type Merlin, or a Merlin-L64P mutant were cultured to confluence. Merlin expression was induced by doxycycline for 2 days. Expression of Merlin was determined by Western blot (left panel). Endogenous YAP was stained and YAP localization was quantified.


A.
Flag-YAP2
Flag-Mst2
HA-Sav
HA-Lats2
HA-Mob
Flag-YAP2
Flag-Mst2
HA-Sav
HA-Lats2
HA-Mob
B.
GST-Sin1
GST-YAP2
HA-Lats2
- WT KR WT
32P-YAP2
WB: GST
WB: HA
32P-Lats2
C.
Relative Luciferase
160
140
120
100
80
60
40
20
0
Gal4-TEAD4
YAP2
Mst2
Sav
Lats2
Mob
D.
Relative Luciferase
160
140
120
100
80
60
40
20
0
Gal4-TEAD4
YAP2
Mst2
Lats2
Mer
Ex
E.
YAP
HA
DAPI
Merge
-
HA-Lats2
HA-Lats2-KR
HA-Mst2
HA-Mst2-KR
HA-Mer
F.
Vec
Mer-WT
Mer-L64P
Vec WT L64P
Merlin
Tubulin
YAP
Nuclear
Cytosolic
Both
14±11%
23±5%
63±6%
10±4%
18±9%
72±8%
21±4%
24±6%
55±4%

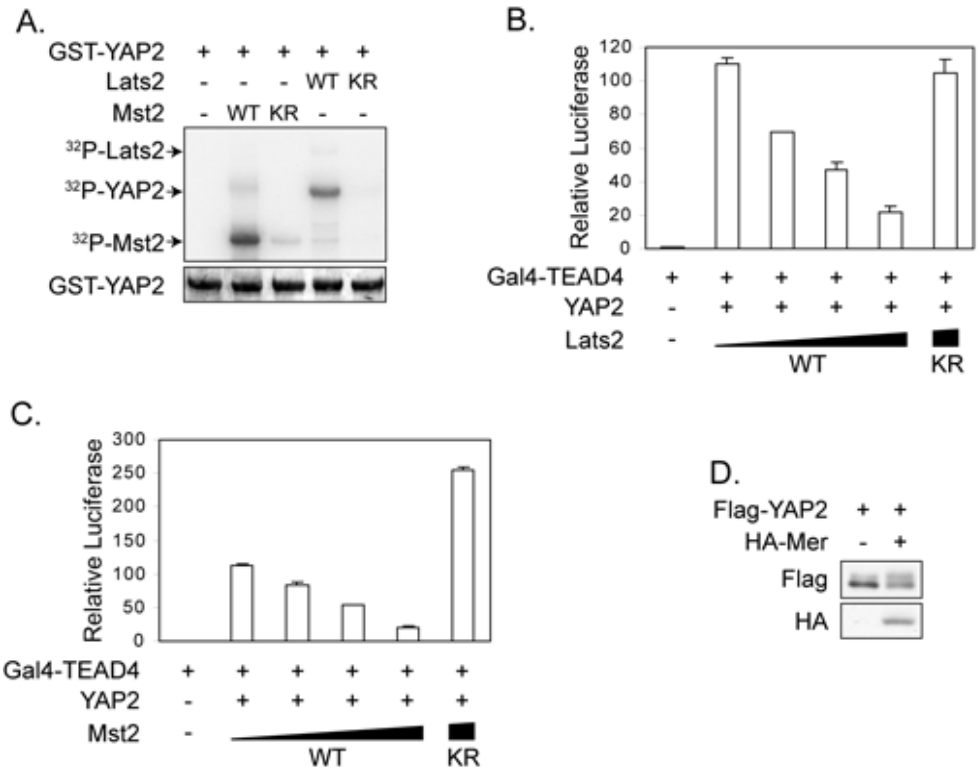


Figure 2.3 Supplemental to the Hippo pathway regulates YAP phosphorylation, activity, and localization.

A. Lats2 but not Mst2 efficiently phosphorylates YAP *in vitro*. Both HA-Mst2 and Lats2 were expressed in HEK293 cells and immunoprecipitated. GST-YAP2 was expressed and purified from *E.coli*. *In vitro* kinase assay reactions of immunoprecipitated HA-Mst2 or HA-Lats2 were performed using GST-YAP2 as substrate in the presence of ^{32}P-ATP. Phosphorylation of proteins *in vitro* was detected by autoradiography after SDS-PAGE (upper panel). The GST-YAP2 input was detected by Coomassie Blue staining (lower panel). KR denotes kinase-inactive mutants of Mst2 or Lats2.

B. The kinase activity of Lats2 is required for inhibition of YAP2 activity. YAP2 activity was assayed based on its ability to co-activate the Gal4 DNA binding domain fused to the TEAD4 transcription factor (Gal4-TEAD4) on the 5×UAS-luciferase reporter in transfected 293T cells. Increasing amount of Lats2 (0, 1, 3, 20ng in each transfection) was co-transfected with the reporter system as indicated. KR denotes the kinase-inactive mutant (30ng). All experiments were triplicated, and luciferase activities were normalized to β-galactosidase activity to control for transfection efficiency.

C. The kinase activity of Mst2 is required for inhibition of YAP2 activity. Experiments are similar to those in panel B except Mst2 was used (0, 10, 20, 50ng wild type and 200ng KR).

D. Merlin co-expression decreases YAP2 mobility.

Lats Inhibits YAP by Phosphorylating HXRXXS Motifs

Lats belongs to the NDR (nuclear Dbf2-related) family of protein kinases (Hergovich et al. 2006). Previous biochemical studies have shown that the yeast Dbf2 kinase recognizes an RXXS motif in its substrates (Mah et al. 2005). Interestingly, in search of such a consensus, we noticed that YAP2 contains five HXRXXS motifs (Fig.2.4A), of which three are conserved in *Drosophila*. It is worth noting that the peptides utilized in elucidating the Dbf2 recognition motif also had a histidine at position -5 (Mah et al. 2005).

We mutated YAP2 by replacing individual serine residues in the HXRXXS motifs with alanine. Among the single mutants tested, S127A, which is conserved in *Drosophila* Yki, was most resistant to Mst2/ Lats2-induced mobility shift (Fig.2.4B). Mutation of all five serine residues (YAP2-5SA) produced a YAP2 downshift more dramatic than that of any single mutant. Furthermore, lambda phosphatase treatment abolished the Mst2/ Lats2-induced mobility shift of YAP2, therefore, verifying the role of phosphorylation in this mobility shift (Fig.2.4B). These results indicate that Ser127 is the primary phosphorylation site in YAP2, while serines in other HXRXXS motifs may also be phosphorylated.

To further confirm the phosphorylation of YAP2 HXRXXS motifs by Lats2, an *in vitro* kinase assay was performed. Mutation of S127 reduced and mutation of all five serine residues abolished YAP2 phosphorylation by Lats2 as determined by ^{32}P incorporation (Fig.2.4C). Phosphorylation of S127 was also verified by immunoblotting with a phospho-YAP (S127) specific antibody (Fig.2.4C). The specificity of this antibody was confirmed by phosphatase treatment (Fig.2.5A). These data demonstrate that Lats2 directly phosphorylates YAP2 on S127 and other serine residues in the HXRXXS motifs.

The functional significance of YAP2 phosphorylation was evaluated by the TEAD4 reporter assay. As shown before, wild type YAP2 was potently inhibited by co-expression of Mst2/Lats2; however, the S127A mutant showed resistance to this

inhibition (Fig.2.4D). Furthermore, the YAP2-5SA mutant was not only resistant to inhibition by Mst2 and Lats2 but also displayed an elevated basal activity (Fig.2.4D). Together, our data demonstrate that YAP2 activity is inhibited by phosphorylation of the HXRXXS motifs, especially S127. Since YAP2-5SA was also partially inhibited by Mst/ Lats, additional levels of regulation may exist.

To confirm the phosphorylation of YAP2 S127 *in vivo* by Lats2, we did co-expression in cultured cells. Co-transfection of Lats2 alone or together with Mst2 increased YAP2 S127 phosphorylation (Fig.2.4E). Interestingly, expression of the inactive Lats2-KR mutant decreased YAP2 basal phosphorylation, perhaps through a dominant negative effect. This supports a role of endogenous Lats in YAP2 phosphorylation. To further confirm this, Lats1 and Lats2 were down-regulated by RNA interference. Knockdown of Lats2 caused a significant reduction in S127 phosphorylation of transfected Flag-YAP2, while knockdown of both Lats1 and Lats2 abolished its phosphorylation (Fig.2.5B). Similarly, knockdown of both Lats1 and Lats2 decreased endogenous YAP phosphorylation (Fig.2.4F), thus establishing an important role of Lats in YAP phosphorylation *in vivo*.

After the determination of Lats target phosphorylation site on YAP, we reexamined the cell density induced phosphorylation of YAP. Along with the reduced electrophoretic migration of YAP as shown in Fig.2.1C, we also observed that YAP S127 phosphorylation was increased by cell density in both NIH-3T3 and MEF cells (Fig.2.4G). To determine whether cell density regulates Lats kinase activity, we immunoprecipitated endogenous Lats2 from NIH-3T3 cells and measured its kinase activity towards YAP *in vitro*. Interestingly, Lats2 from high density culture displayed an elevated activity (Fig.2.4H). This result directly suggests the activation of Lats and possibly the Hippo pathway under high cell density, which nicely explains the increased phosphorylation of YAP.

It has been previously reported that YAP2 S127 is phosphorylated by Akt/PKB (Basu et al. 2003). However, the reported YAP inhibition by Akt dependent phosphorylation is inconsistent with recent genetic data that demonstrate YAP as an

oncogene. We tested the function of Akt in YAP phosphorylation. Surprisingly, neither LY294002 nor wortmannin (two PI3K inhibitors) decreased YAP2 S127 phosphorylation, although they potently blocked the phosphorylation of Akt and GSK3, a physiological Akt substrate (Fig.2.6A). In addition, neither EGF nor insulin stimulated YAP2 phosphorylation, while both strongly stimulated the phosphorylation of Akt and GSK3 (Fig.2.6A, 2.6B). Phosphorylation of Akt T308 by PDK1 is essential for Akt activity (Williams et al. 2000). However, YAP phosphorylation was not affected by PDK1 knockout (Fig.2.6C). In addition, co-expression of wild type or constitutively active myristoylated Akt did not increase YAP2 phosphorylation (Fig.2.6D). We also observed that Akt did inhibit YAP2 activity but in a kinase activity independent manner, suggesting that Akt overexpression could inhibit YAP2 indirectly (Fig.2.6E). Together, our results demonstrate that Akt is unlikely to be responsible for YAP2 S127 phosphorylation.

Figure 2.4 Lats inhibits YAP by phosphorylating HXRXXS motifs.

A. YAP2 contains five HXRXXS motifs. The yeast Dbf2 optimal target sequence was aligned with the five HXRXXS motifs of human YAP2.

B. Serine 127 is the major phosphorylation site in YAP2. Wild type or mutant Flag-YAP2 was co-transfected with HA-Mst2 and HA-Lats2 as indicated. YAP2 mobility shift was determined by anti-Flag Western blot.

C. Lats2 directly phosphorylates YAP2 on HXRXXS motifs. In vitro phosphorylation of YAP2 mutants with immunoprecipitated HA-Lats2 was performed. Phosphorylation of GST-YAP2 was detected by either 32P incorporation or anti-phospho-YAP (S127) Western blot. GST-YAP2 input was shown by Coomassie Blue staining (lower panel).

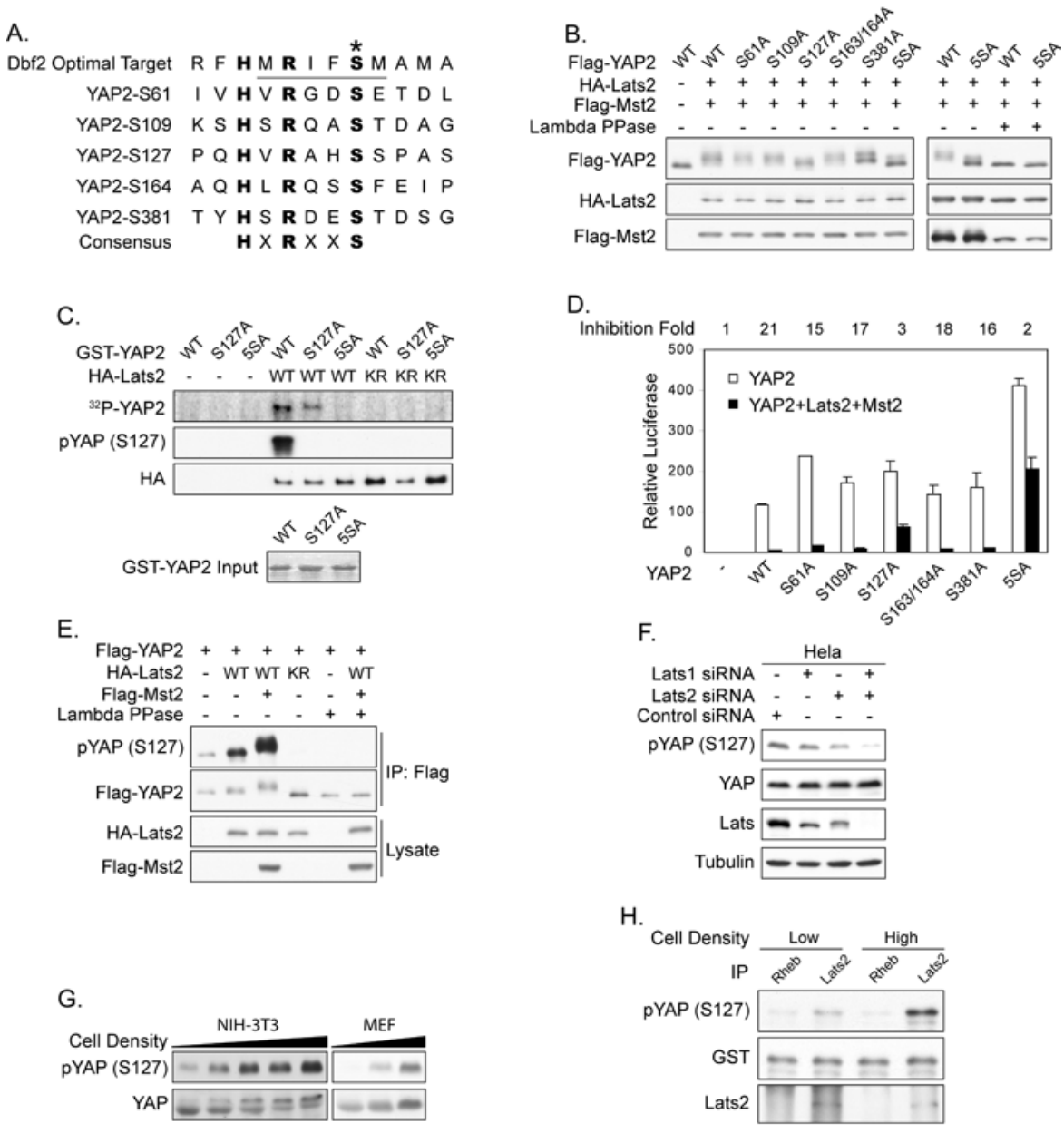
D. YAP2 phosphorylation defective mutants S127A and 5SA are resistant to inhibition by Mst2 and Lats2. The reporter assay is similar to those in Fig.2.2C. The fold activity inhibition of each mutant by Mst2/ Lats2 is indicated at the top of this panel.

E. Co-expression of Mst2 and Lats2 increases YAP2 S127 phosphorylation. Flag-YAP2 was co-transfected with HA-Lats2 and Flag-Mst2 into HEK293 cells as indicated. Flag-YAP2 was immunoprecipitated and phosphorylation of S127 was detected by pYAP (S127) antibody.

F. Knockdown of Lats decreases endogenous YAP S127 phosphorylation. HeLa cells were transfected twice with siRNA for Lats1 and Lats2 as indicated. Phosphorylation and protein levels of endogenous YAP were determined by Western blot. Knockdown of Lats was verified by the anti-Lats antibody, which recognizes both Lats1 and Lats2.

G. YAP S127 phosphorylation increases with cell density. NIH-3T3 and MEF cells were harvested at different densities, and YAP phosphorylation was assayed.

H. Lats2 kinase activity increases with cell density. NIH-3T3 cells were harvested at different densities. Endogenous Lats2 was immunoprecipitated and used in an in vitro kinase assay. Phosphorylation of GST-YAP2 was detected by anti-phospho-YAP (S127) Western blot. Rheb IP was included as a negative control.


A.
Dbf2 Optimal Target R F H M R I F S M A M A
YAP2-S61 I V H V R G D S E T D L
YAP2-S109 K S H S R Q A S T D A G
YAP2-S127 P Q H V R A H S S P A S
YAP2-S164 A Q H L R Q S S F E I P
YAP2-S381 T Y H S R D E S T D S G
Consensus H X R X X S
B.
Flag-YAP2 WT WT S61A S109A S127A S163/164A S381A 5SA WT 5SA WT 5SA
HA-Lats2
Flag-Mst2
Lambda PPase
Flag-YAP2
HA-Lats2
Flag-Mst2
C.
GST-YAP2 WT S127A 5SA WT S127A 5SA WT S127A 5SA
HA-Lats2 - - - WT WT WT KR KR KR
32P-YAP2
pYAP (S127)
HA
GST-YAP2 Input
D.
Inhibition Fold 1 21 15 17 3 18 16 2
Relative Luciferase
YAP2
YAP2+Lats2+Mst2
YAP2 WT S61A S109A S127A S163/164A S381A 5SA
E.
Flag-YAP2
HA-Lats2 - WT WT KR - WT
Flag-Mst2
Lambda PPase
pYAP (S127)
Flag-YAP2
IP: Flag
HA-Lats2
Flag-Mst2
Lysate
F.
Hela
Lats1 siRNA
Lats2 siRNA
Control siRNA
pYAP (S127)
YAP
Lats
Tubulin
G.
NIH-3T3
MEF
Cell Density
pYAP (S127)
YAP
H.
Cell Density Low High
IP Rheb Lats2 Rheb Lats2
pYAP (S127)
GST
Lats2

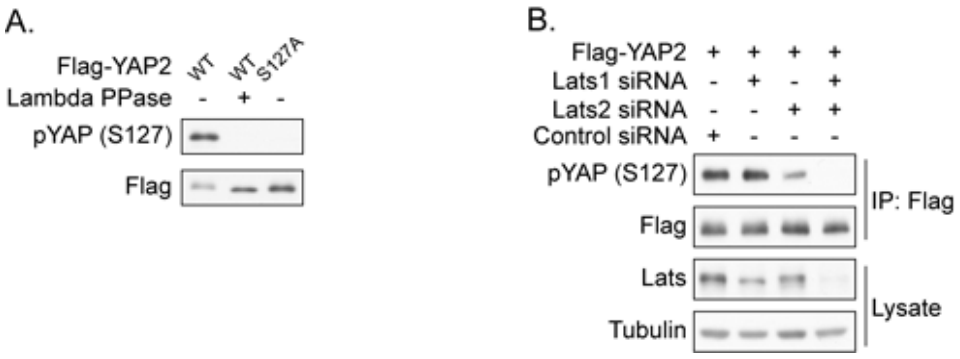


Figure 2.5 Supplemental to Lats inhibits YAP by phosphorylating HXRXXS motifs.

A. The anti-phospho-YAP (S127) antibody specifically detects serine 127 phosphorylated YAP. HEK293 cells were transfected with Flag-YAP2 WT or S127A mutant. Flag-YAP2 was immunoprecipitated and lambda phosphatase treated as indicated. pYAP (S127) antibody was used to detect the presence of phosphorylated Flag-YAP2. Mutation of S127 or lambda phosphatase treatment completely abolished the recognition of YAP2 by the pYAP(S127) antibody.

B. Lats1 and Lats2 are required for YAP2 S127 phosphorylation. Flag-YAP2 was co-transfected with siRNA for Lats1 and Lats2 as indicated. Flag-YAP2 was then immunoprecipitated and S127 phosphorylation was examined. Knockdown of Lats was verified by the anti-Lats antibody, which recognizes both Lats1 and Lats2.

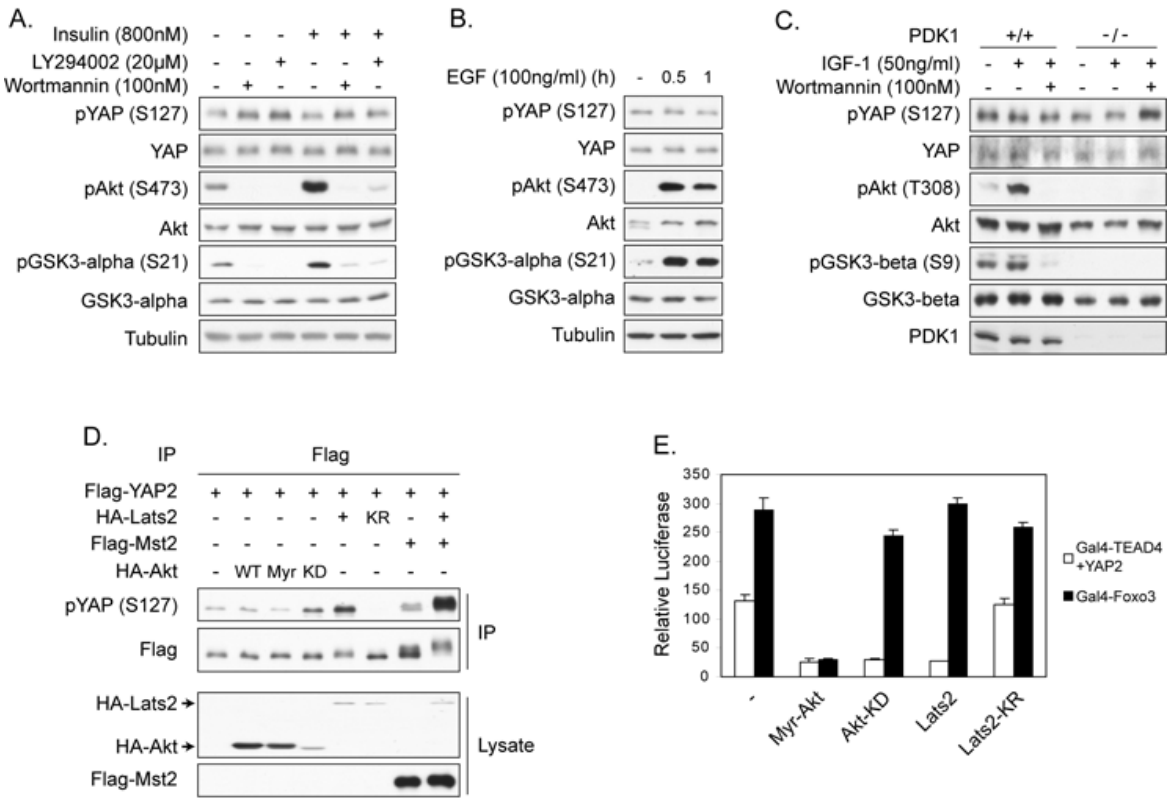


Figure 2.6 Akt does not phosphorylate YAP2 S127.

A. Insulin activates Akt but not YAP S127 phosphorylation. HeLa cells were treated with LY294002 or wortmannin for one hour and insulin for 30 min as indicated. Protein levels and phosphorylation of YAP, Akt, and GSK3α were determined using respective antibodies.

B. EGF stimulates Akt but not YAP S127 phosphorylation. HeLa cells were serum starved for 24 hours and treated with 100ng/ml EGF for the indicated time. Protein levels and phosphorylation of YAP, Akt, and GSK3α were determined using respective antibodies.

C. PDK1 knockout does not affect YAP S127 phosphorylation. PDK1 +/+ or -/- ES cells were serum starved for 3 hours followed by treatment with wortmannin for one hour and IGF-1 for 30 min as indicated. Western blots were done as indicated.

D. Co-expression of Lats2 but not Akt promotes YAP2 S127 phosphorylation. Flag-YAP2 was co-transfected with various plasmids as indicated into HEK293 cells. HA-Akt-WT, Myr, and KD denote wild type, myristoylated (constitutively active), and kinase dead mutant of Akt, respectively. Flag-YAP2 was immunoprecipitated and phosphorylation of S127 was determined by phosphoYAP (S127) Western blot.

E. The kinase activity of Lats2 but not Akt is required to inhibit YAP2. Experiments were similar to those in Fig.2.3B. Open columns and filled columns denote the reporter assays for YAP2 and Foxo3 (a functional target of Akt), respectively.

Phosphorylation Promotes YAP Cytoplasmic Localization and Inhibits its Transcription Factor Binding

To directly determine the effect of phosphorylation on YAP localization, we performed immunofluorescence staining of transfected wild type or phosphorylation-deficient YAP2. Flag-YAP2 showed prominent nuclear localization in transfected cells, while co-expression of Lats2 induced nearly complete cytoplasmic translocation (Fig.2.7A). Interestingly, Lats2 had only minor effects on YAP2-S127A and 5SA localization.

To determine whether Lats-dependent phosphorylation is indeed responsible for YAP translocation under high cell density, we examined MCF10A cells stably expressing Myc-YAP2 or Myc-YAP2-5SA. Similar to endogenous YAP, Myc-YAP2 showed density dependent subcellular localization (Fig.2.7B). In contrast, Myc-YAP2-5SA displayed both nuclear and cytoplasmic staining under high density. Together, our studies suggest that phosphorylation of HXRXXS motifs by Lats is at least in part responsible for the nuclear-to-cytoplasm translocation of YAP in response to cell contact signals.

YAP is a transcription co-activator, therefore, we hypothesized that the Lats induced cytoplasmic translocation of YAP inhibits its function by attenuating its interaction with nuclear-localized transcription factors. Indeed, we observed that co-expression of Mst2 and Lats2 decreased the association between TEAD4 and YAP2 (Fig.2.8) but had no effect on the interaction between TEAD4 and YAP2-S127A or 5SA (Fig.2.7C). To exclude the possibility that YAP2 phosphorylation directly affects YAP2/TEAD4 interaction affinity, we tested whether dephosphorylation affects YAP2/ TEAD4 association *in vitro*. Immunoprecipitated YAP2 was treated with lambda phosphatase and incubated with Myc-TEAD4 containing cell lysate. As shown in Fig.2.7D, dephosphorylation of YAP2 had little effect on its interaction with TEAD4 *in vitro*. Therefore, we conclude that YAP2 phosphorylation by Lats2 leads to decreased interaction with TEAD secondary to cytoplasmic retention.

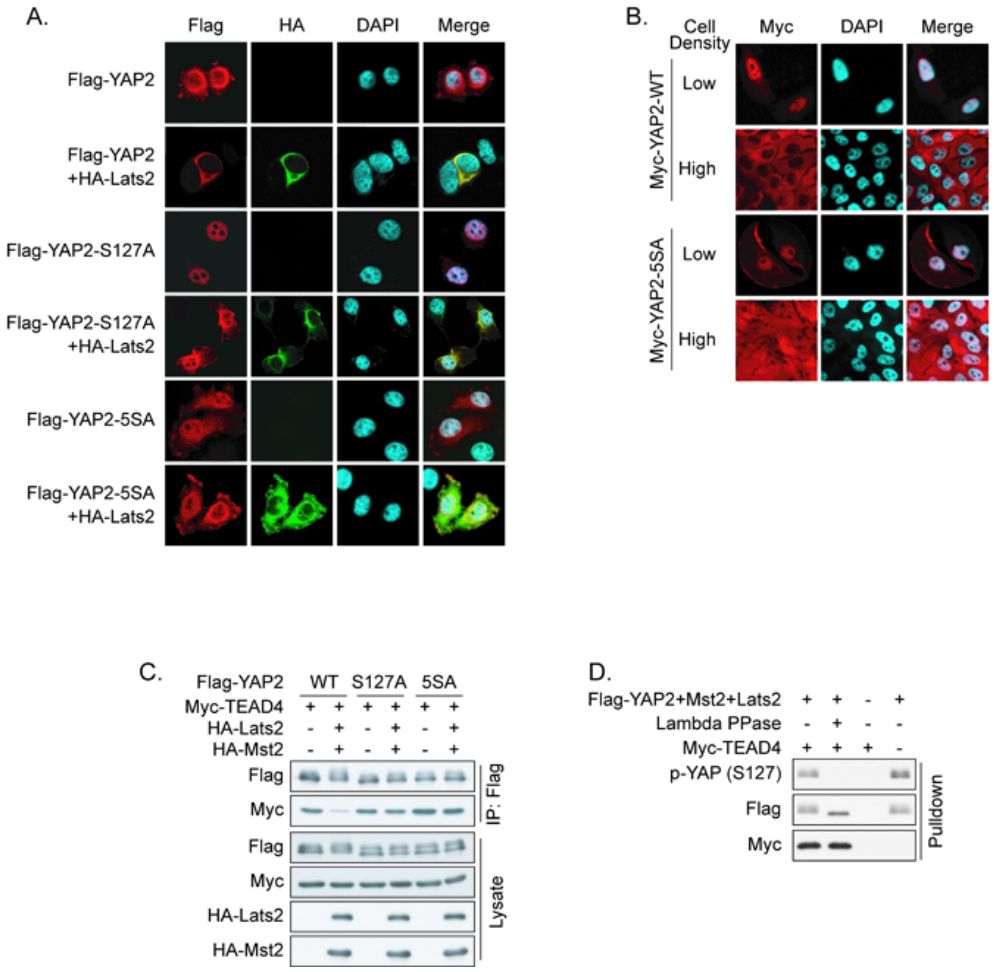


Figure 2.7 Phosphorylation promotes YAP cytoplasmic localization and inhibits transcription factor binding.

A. Serine 127 is required for YAP2 cytoplasmic localization induced by Lats2. Flag-YAP2 wild type or mutants were transfected alone or together with HA-Lats2 into HeLa cells. Cells were stained with Flag and HA antibodies.

B. Phosphorylation is required for cell density-induced YAP2 cytoplasmic translocation. MCF10A cells stably expressing Myc-YAP2 or Myc-YAP2-5SA were cultured at low or high density. Myc-YAP2 was stained with anti-Myc antibody.

C. Lats and Mst decrease YAP2/TEAD4 interaction *in vivo* in a S127 dependent manner. Indicated plasmids were transfected into HEK293 cells. Flag-YAP2 was immunoprecipitated, and co-precipitated Myc-TEAD4 was detected by Western blot.

D. YAP2 dephosphorylation does not affect its interaction with TEAD4 *in vitro*. Flag-YAP2 (co-transfected with Mst2 and lats2) immunoprecipitated from HEK293 cells were treated with lambda phosphatase as indicated and then used in an *in vitro* TEAD pull-down assay. Myc-TEAD4 was prepared from transfected HEK293 cells. The final products were analyzed by Western blot.

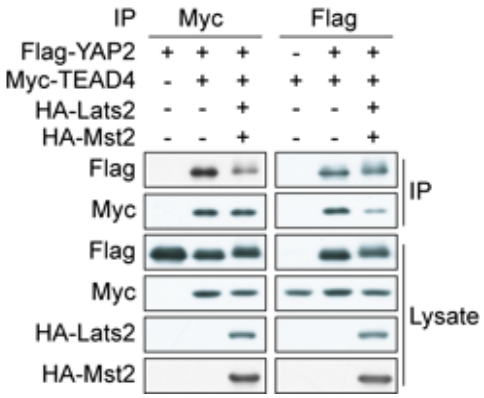


Figure 2.8 Mst2 and Lats2 co-expression decreases YAP2 and TEAD4 interaction.

Transfection of various plasmids into HEK293 cells is indicated. Myc-TEAD4 (left panel) or Flag-YAP2 (right panel) was immunoprecipitated, and the immunoprecipitates were probed with specific antibodies as indicated.

S127 Phosphorylation Regulates YAP and 14-3-3 Interaction

One commonly seen mechanism of cytoplasmic retention of nuclear proteins is 14-3-3 binding (Muslin and Xing 2000). Interestingly, YAP S127 phosphorylation has been reported to create a 14-3-3 binding site (Basu et al. 2003). We observed that YAP2 interacts with 14-3-3, and this interaction is completely abolished by phosphatase treatment (Fig.2.9A). Furthermore, expression of Mst2 and Lats2 but not Akt increased the interaction between YAP2 and 14-3-3 in a S127-dependent manner, as neither YAP2-S127A nor 5SA showed any binding to 14-3-3 (Fig.2.9B). Our results suggest a model in which Lats2 promotes YAP2 cytoplasmic localization by increasing S127 phosphorylation and 14-3-3 binding.

Study of the yeast Dbf2 kinase has shown that R at the -3 position of target S/T is critical for kinase recognition (Fig.2.4A). However the function of the H at the -5 position is unknown. We tested the importance of this histidine by examining the phosphorylation of YAP2-H122Y and H122L mutants. Mutation of H122 to either Y or L significantly decreased S127 phosphorylation *in vitro* and *in vivo* (Fig.2.9C, 2.9D), indicating the importance of the histidine at the -5 position. These two mutations also attenuated interaction with 14-3-3 (Fig. 2.9D), which is likely due to decreased S127 phosphorylation. A proline at the +2 position of a phosphorylated serine is critical for 14-3-3 binding. To investigate the importance of this proline, we assayed 14-3-3 interaction with a YAP2-P129D mutant. As expected, mutation of P129 completely eliminated 14-3-3 binding and also decreased recognition by the pYAP antibody (Fig.2.9E). However, *in vitro* phosphorylation assays showed that the P129D mutation did not affect phosphorylation by Lats2 (Fig.2.9C). Based on the above data, we conclude that P129 is important for 14-3-3 binding but is not directly involved in YAP phosphorylation by Lats. In contrast, H122 plays a critical role in YAP phosphorylation by Lats.

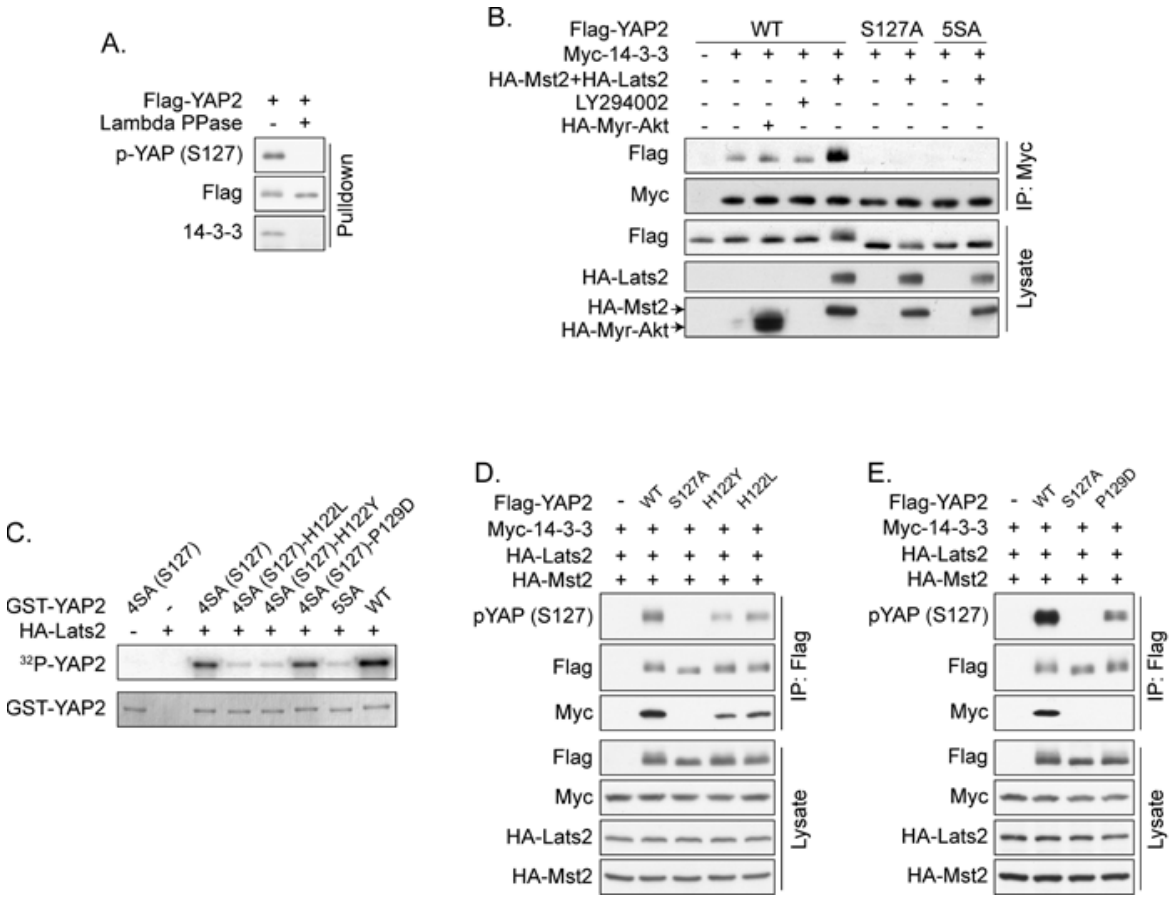


Figure 2.9 S127 phosphorylation regulates YAP and 14-3-3 interaction.

A. Dephosphorylation abolishes the interaction between YAP2 and 14-3-3 *in vitro*. Flag-YAP2 immunoprecipitated from transfected HEK293 cells was treated with lambda phosphatase as indicated and then used to pulldown endogenous 14-3-3 from HEK293 cell lysate. The products were analyzed by Western blot.

B. Lats2 but not Akt enhances YAP2 and 14-3-3 interaction. Flag-YAP2 plasmids were co-transfected with Myc-14-3-3 and other indicated plasmids into HEK293 cells. Myc-14-3-3 was immunoprecipitated and co-immunoprecipitated Flag-YAP2 was detected.

C. Mutation of H122 but not P129 decreases YAP2 S127 phosphorylation by Lats2. *In vitro* phosphorylation of YAP2 mutants by immunoprecipitated HA-Lats2 was performed. Phosphorylation of GST-YAP2 was detected by ^{32}P incorporation. GST-YAP2 input was shown by Coomassie Blue staining (lower panel). 4SA (S127) denotes that 4 of the 5 Lats phosphorylation sites were mutated to alanine except serine 127.

D. Mutation of histidine 122 in YAP2 impairs serine 127 phosphorylation and 14-3-3 binding. Indicated plasmids were transfected into HEK293 cells. Flag-YAP2 was immunoprecipitated, and the immunoprecipitates were probed as indicated.

E. Proline 129 of YAP2 is required for 14-3-3 binding. Experiments were similar as panel D.

S127 Phosphorylation Regulates YAP and Yki Biological Function

In a genetic screen for suppressors of phenotypes caused by Hippo overexpression, we recovered three alleles of *yki*. Remarkably, our *yki* alleles all affect the highly conserved region surrounding S168, which corresponds to S127 in YAP2. As shown in Fig.2.10A, two of the alleles harbor mutations of H163 to Y and L, while the other allele harbors a mutation of P170 to S. All three alleles strongly suppress the small wing phenotype caused by Hippo overexpression and exhibit weak semi-dominant phenotypes (Fig.2.10B, panels a-d). The most noticeable dominant phenotypes were in the wings which were slightly larger and often had defects in the posterior crossvein. Due to the large wing phenotypes, we named these *yki* alleles after the large ears of the Dumbo cartoon character. The suppression of Hippo hyperactivated phenotypes suggests that these mutant Yki proteins are constitutively active and evade suppression by Hippo signaling, which is consistent with our biochemical studies of YAP2. If this were the case we would expect that cells homozygous mutant for these *yki* alleles phenocopy *hippo* loss-of-function mutations. Indeed, we found that yki^{Dbo} mutant cells had a growth advantage over wild type cells (Fig.2.10B, e-h). This effect was apparent in adult eyes showing that yki^{Dbo} mutant cells, marked by the lack of pigmentation, out-competed red wild type cells (Fig.2.10B, e-f) as well as in developing eye tissues at larval stages, where *eyFLP* induced yki^{Dbo} mutant clones occupied nearly the entire disc tissues in contrast to wild type control clones which occupied less than half of the discs (Fig.2.10B, g-h). In addition, yki^{Dbo} mutant eye tissue exhibited ectopic cell proliferation posterior to the morphogenetic furrow (Fig.2.10B, i, i'), a region where wild type cells exit the cell cycle and start to differentiate, and produced an excess number of interommatidial cells (Fig.2.10B, j, j'). Characteristic for mutations in Hippo signaling components, *cyclin E* and *ex*, transcriptional targets of Hippo signaling, were up-regulated in yki^{Dbo} clones (Fig.2.10B, k-l). Interestingly, this up-regulation was also observed in heterozygous cells, perhaps due to the semi-dominant nature of the yki^{Dbo} alleles.

The combination of these phenotypes is very distinctive for loss of Hippo signaling, although they are not as severe as those observed for null mutants of *hippo* or *wts*. The *yki*^{Dbo} alleles thus mimic hypomorphic *hippo* alleles. We conclude that the *Yki*^{Dbo} mutations produce dominant active proteins that are not as efficiently suppressed by Hippo signaling. This is likely due to reduced phosphorylation by Wts (Yki-*Dbo1* and *Dbo2*) and reduced 14-3-3 binding (Yki-*Dbo3*), as observed for the respective YAP mutants.

In another line of evidence, we compared the activity of YAP/Yki and the phosphorylation-deficient S127A mutant in transgenic flies that overexpressed these proteins in developing eyes. As expected, over-expression of YAP2 or Yki increased the transcription of *diap1-lacZ* (Fig.2.10C, panels a-d) and *CycE-lacZ* (Fig.2.11, panels a-d) reporter genes, transcriptional readouts for Yki activity (Huang et al. 2005). Overexpression of YAP2 had a moderate effect on eye size and slightly increased the size of larval eye discs and adult eyes (Fig.2.10C, panels f, j). The phosphorylation-defective YAP2-S127A was more potent and caused a significant increase in the size of eye discs and in the number of interommatidial cells (Fig.2.10C, panels g, k). The adult eyes of such animals were overgrown but folded and had severe morphological defect (Fig.2.10C, panels a-c, i-k). All these phenotypes are reminiscent of *warts* and *mats* mutants and the YAP2-S127A was in fact as potent as the fly Yki protein in promoting tissue growth (Fig.2.10C). These data together with ones from the *Dbo* mutants, suggest a critical role of YAP/Yki phosphorylation by Lats/Wts in the negative regulation of YAP/Yki *in vivo*.

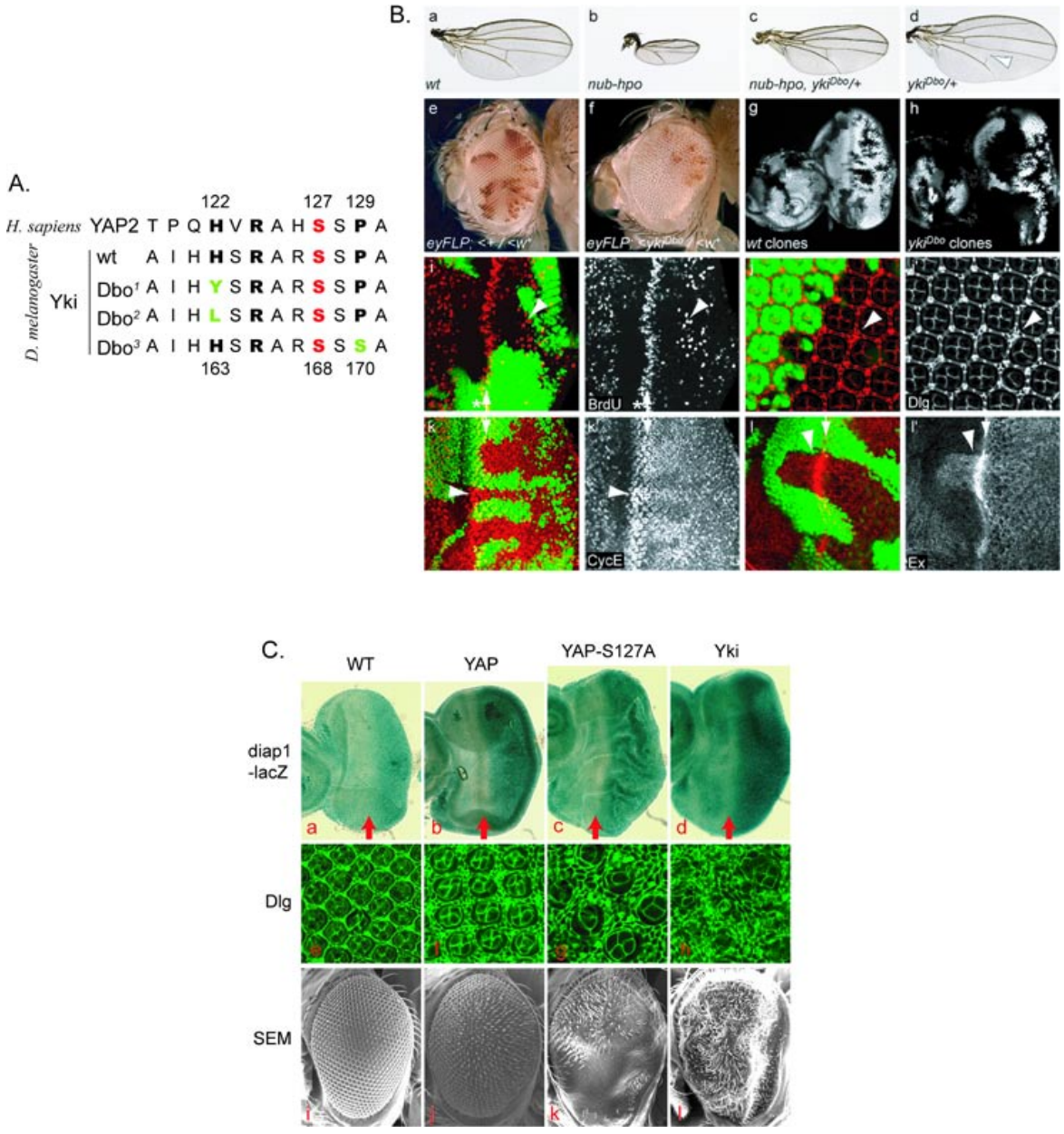
Figure 2.10 S127 phosphorylation regulates YAP and Yki biological function *in vivo*.

A. Alignment of the *H. sapiens* YAP2 and the *D. melanogaster* Yki wild-type and Dbo mutant proteins around the S127 (YAP2) residue. Mutated residues are shown in green.

B. Dominant active *yorkie* mutations around the phosphorylation site S168 mimic *hippo* loss-of-function phenotypes. **(a)** wild-type wing. **(b)** Hpo overexpression driven by *nubbin-Gal4*. **(c)** *nubbin-Gal4 UAS-Hpo, yki*^{Dbo}/+. **(d)** *yki*^{Dbo}/+. **(e)** A fly with an eye mosaic for a mutation in the *white* gene. Clones were induced using the eye-specific FLP driver (*eyFLP*), and a cell-lethal mutation on the homologous [w^{+}] chromosome was used to eliminate twin spot clones, which increased the area of the *w*$^{-}$ cell clones. **(f)** A fly with a mosaic eye induced by the same method as in (e). However, this fly carries a *yki*^{Dbo} mutation on the *w*$^{-}$ chromosome. **(g, h)** Eye imaginal discs from third-instar larvae containing *wt* and *yki*^{Dbo} mutant clones that were marked by the absence of GFP (gray). **(i-l)** *yki*^{Dbo} mutant clones marked by the absence of GFP. **(i)** Eye imaginal disc containing *yki*^{Dbo} mutant clones and labeled for BrdU incorporation (red in [i] and grayscale in [i']). Asterisks indicate the morphogenetic furrow, arrows indicate the second mitotic wave, and arrowheads point to ectopic cell proliferation in *yki*^{Dbo} mutant clones posterior to the second mitotic wave. **(j)** Mid-pupal retina stained with Discs large (Dlg) antibodies to visualize cell outlines (red in [j] and gray scale in [j']). *yki*^{Dbo} mutant clones showed extra interommatidial cells (arrowhead). **(k)** *yki*^{Dbo} mutant clones showed upregulated expression of Cyclin E (arrowheads), red in [k] and grayscale in [k'], most conspicuously behind the second mitotic wave (arrows). **(l)** *yki*^{Dbo} mutant clones showed increased Ex (red in [l] and grayscale [l']) levels in the eye imaginal disc.

C. The phosphorylation defective YAP2-S127A is more active in promoting tissue growth in *Drosophila*. Third instar larval eye discs were analyzed for the transcriptional activities of *diap1-lacZ* (a-d) reporter genes. Anterior is to the left. Red arrows indicate the morphogenetic furrow. Mid-pupal eye discs were stained with Discs large (Dlg) antibody to outline cells (e-h). SEM (scanning electron microscopy) images of fly adult eyes are presented in (i-l). Genotypes of the fly tissues are:

(a). *GMR-Gal4/+; diap1-lacZ/+*
(b). *GMR-Gal4/UAS-Flag-YAP2; diap1-lacZ/+*
(c). *GMR-Gal4/UAS-Flag-YAP2*^{S127A}*; diap1-lacZ/+*
(d). *GMR-Gal4/UAS-yki-V5; diap1-lacZ/+*
(e, i). Wild-type (Canton S)
(f, j). *GMR-Gal4/UAS-Flag-YAP2*
(g, k). *GMR-Gal4/UAS-Flag-YAP2*^{S127A}
(h, l). *GMR-Gal4/UAS-yki-V5*


A.
122 127 129
H. sapiens YAP2 T P Q H V R A H S S P A
D. melanogaster
Yki
wt A I H H S R A R S S P A
Dbo1 A I H Y S R A R S S P A
Dbo2 A I H L S R A R S S P A
Dbo3 A I H H S R A R S S S A
163 168 170
B.
a
b
c
d
wt
nub-hpo
nub-hpo, yki^Dbo/+
yki^Dbo/+
e
f
g
h
eyFLP; <+ / <w+
eyFLP; <yki^Dbo / <w+
wt clones
yki^Dbo clones
i
j
k
l
BrdU
Dlg
k'
l'
CycE
Ex
C.
WT
YAP
YAP-S127A
Yki
diap1
-lacZ
Dlg
SEM
a
b
c
d
i
j
k
l

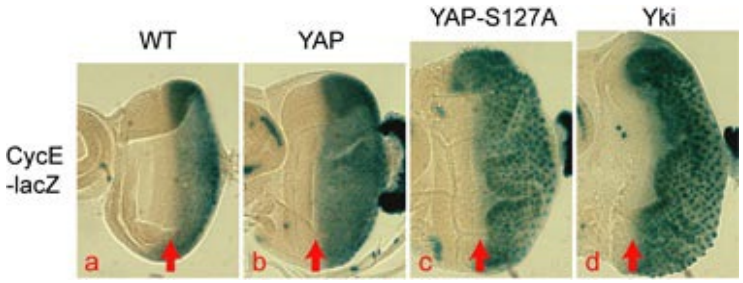


Figure 2.11 The phosphorylation defective YAP2-S127A is more active in promoting *CycE* expression in *Drosophila*.
Third instar larval eye discs were analyzed for the transcription activities of *CycE-lacZ* (a-d) reporter genes. The anterior is to the left. Red arrows indicate the morphogenetic furrow.
(a). *GMR-Gal4/+; CycE-lacZ/+*
(b). *GMR-Gal4/UAS-Flag-YAP2; CycE-lacZ/+*
(c). *GMR-Gal4/UAS-Flag-YAP2^{S127A}; CycE-lacZ/+*
(d). *GMR-Gal4/UAS-yki-V5; CycE-lacZ/+*

YAP Regulates Density-dependent Gene Expression and Alteration of YAP Activity Affects Cell Contact Inhibition

As a transcription co-activator, YAP functions by regulating gene expression. Gene expression microarray experiments were performed to compare genes that are regulated by YAP and by cell density. We found that the set of genes induced by YAP2 significantly overlaps with the set of genes that are repressed by high cell density (Fig.2.12A). Similarly, the set of genes repressed by YAP2 (possibly by indirect means) significantly overlaps with the set of genes induced by high density. However, the set of genes induced (or repressed) by YAP2 does not significantly overlap with the set of genes induced (or repressed) by high density. The opposite regulation of gene expression by YAP and high cell density was confirmed by quantitative RT-PCR of selected genes (Fig.2.12B). These observations indicate that YAP and cell density regulate many genes in opposite manners.

Our data indicates that YAP may play a role in cell contact inhibition. To further investigate YAP regulation by cell contact, scratch wounds were generated in confluent cell cultures to relieve contact inhibition. As shown in Fig.2.12C, both YAP staining intensity and nuclear localization were significantly elevated in cells at the border of the wound, while cells further away showed cytoplasmic localization of YAP. Interestingly, the nuclear YAP-positive cells were also positive for Ki67, a marker of cell proliferation, indicating that these cells have re-entered the cell division cycle. The above data further demonstrates that YAP localization is regulated by cell density and that nuclear YAP may promote cell cycle entry.

To test the effect of increased YAP activity on contact inhibition, we stably expressed YAP2 in NIH-3T3 cells. YAP2-overexpressing NIH-3T3 cells kept proliferating even after reaching confluency and resulted in a higher saturation density than vector control cells (Fig.2.12D). Confluent cells were also analyzed for cell cycle distribution by BrdU incorporation assay. Many more YAP2-expressing cells (23%) were still in S-phase compared with vector control cells (6%) (Fig.2.12E). The above observations suggest that YAP overexpression may overcome contact inhibition, and

therefore further supports an important role of YAP inactivation by the Hippo pathway in cell contact inhibition.

We tested if interference of YAP activity were able to restore contact inhibition in cancer cells that are otherwise deficient of it. ACHN is a cancer cell line from a metastatic human renal adenocarcinoma. It has been reported to bear a deletion of Sav (Tapon et al. 2002), which suggests a deficient Hippo pathway. Consistently, this cell line clearly growing on top of each other and pile up even under low cell density (Fig. 2.12F), suggesting lose of contact inhibition. We generated ACHN cells stably express YAP2-5SA-ΔC, which is the YAP2-5SA nucleus-localizing form with a deletion of the C-terminal transcription activation domain. This mutant YAP2 is insensitive to the Hippo pathway induced cytoplasmic translocation and ccould not activate gene expression, therefore may act as a dominant-negative form. Although the expression of this mutant YAP2 was as low as endogenous YAP (Fig. 2.12F), its effect was dramatic. The YAP2-5SA-ΔC expressing ACHN cells grow as a single layer and do not pile up even after confluent (Fig. 2.12F). This result indicates at least in Hippo pathway deficient cancer cells, that the loss of cell contact inhibition can be restored by blocking endogenous YAP function, and therefore further supports the function of YAP in contact inhibition.

Figure 2.12 YAP regulates density-dependent gene expression and alteration of YAP activity affects cell contact inhibition.

A. High cell density and YAP affect gene expression in an opposite manner. YAP regulated genes were revealed by microarray analyses of control and YAP overexpressing NIH-3T3 cells. Density-regulated genes were also identified by microarray analysis of sparse and confluent cells. Genes that show more than two-fold differences were used in the comparison. *P* values were calculated by Fisher exact test.

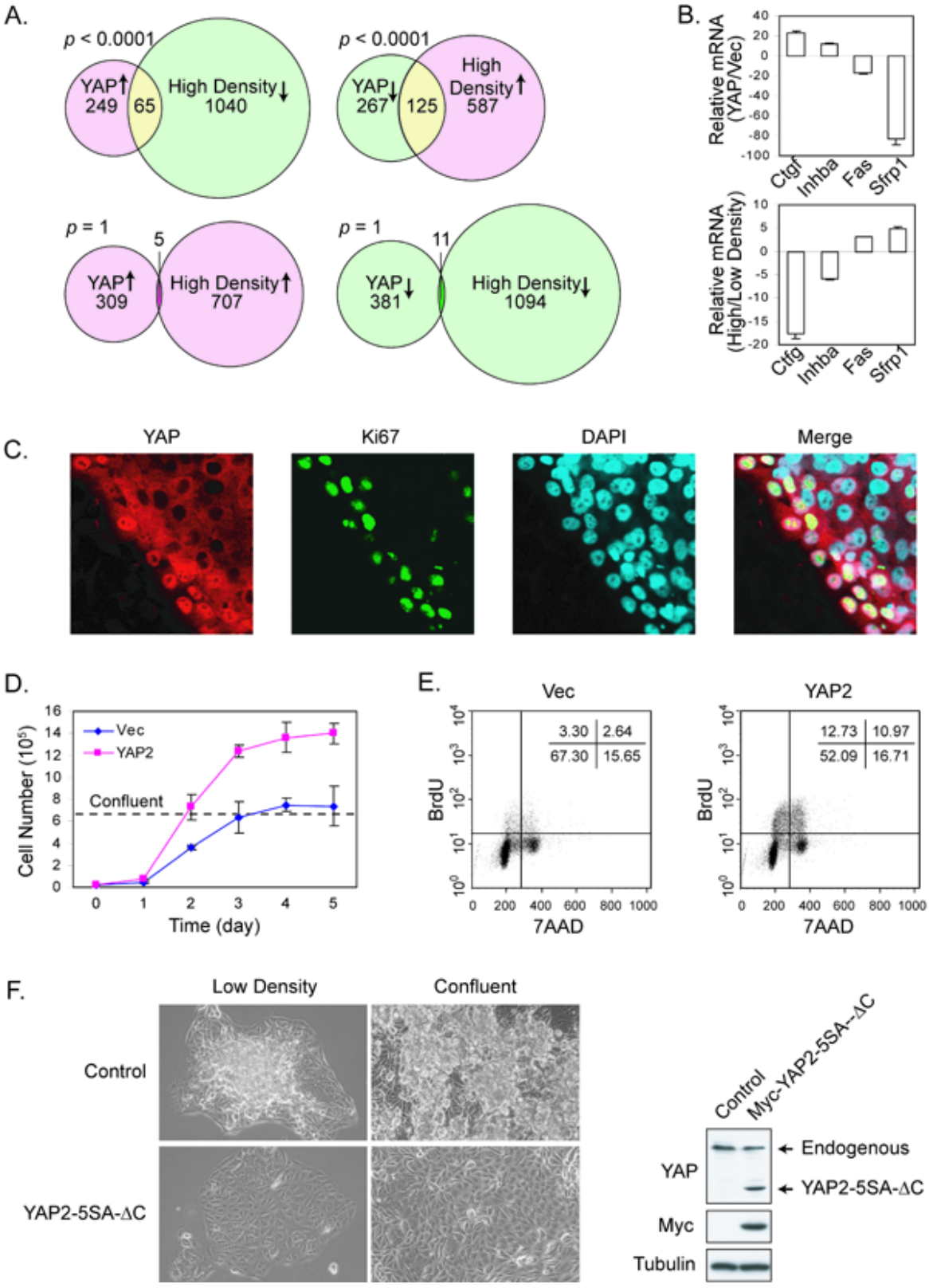
B. Quantitative RT-PCR confirmation of YAP and cell density regulated genes. Total RNA isolated from NIH-3T3 cells stably expressing YAP2 or vector control (upper chart) and from low or high density cultures (lower chart) were analyzed by quantitative RT-PCR and normalized to HPRT (hypoxanthine phosphoribosyltransferase 1).

C. Correlation of cell proliferation and nuclear YAP localization. Confluent MCF10A culture was scratched. Six hours later, cells were fixed and stained for YAP and Ki67.

D. YAP promotes cell growth and elevates saturation density. Growth curves of NIH-3T3 cells stably expressing YAP2 or vector were determined. Confluent density is indicated.

E. YAP promotes proliferation of confluent cells. Vector and YAP overexpressing NIH-3T3 cells were cultured to confluence. Cells at a similar density were pulse labeled with BrdU followed by staining with anti-BrdU and 7-AAD (a fluorescent dye for total DNA) for flow cytometric analysis.

F. Dominant-negative YAP restores contact inhibition in ACHN cancer cells. ACHN cells stably expressing vector or Myc-YAP2-5SA-ΔC were cultured to low density or confluence. Cell morphologies are shown in the left panels. The loss of contact inhibition in ACHN cells is evident that cells pile on top of each other. Myc-YAP2-5SA-ΔC expression level is shown by Western blot in the right panels.


A.
p < 0.0001
YAP↑ 249
65
High Density↓ 1040
p < 0.0001
YAP↓ 267
125
High Density↑ 587
p = 1
5
YAP↑ 309
High Density↑ 707
p = 1
11
YAP↓ 381
High Density↓ 1094
B.
Relative mRNA (YAP/Vec)
Ctgf
Inhba
Fas
Sfrp1
Relative mRNA (High/Low Density)
C.
YAP
Ki67
DAPI
Merge
D.
Cell Number (10^5)
Vec
YAP2
Confluent
Time (day)
E.
Vec
YAP2
BrdU
7AAD
3.30
2.64
67.30
15.65
12.73
10.97
52.09
16.71
F.
Low Density
Confluent
Control
YAP2-5SA-ΔC
Control
Myc-YAP2-5SA-ΔC
YAP
Endogenous
YAP2-5SA-ΔC
Myc
Tubulin

Elevated YAP Protein and Nuclear Localization in Cancers

Although YAP has been implicated as a candidate oncogene, it has not been reported whether YAP is indeed activated in human cancers. We evaluated YAP expression in human cancers by immunohistochemical staining of tissue microarrays. Among the 115 cases of hepatocellular carcinoma (HCC) samples examined, 63 samples (54%) showed strong YAP staining, while 95% of normal liver tissue samples (40 out of 42 cases) showed very weak staining, indicating a significant difference in YAP protein levels between normal and cancerous tissues ($p<0.001$, Fisher exact test) (Fig.2.13A, 2.13B). Furthermore, the majority of HCC cells displayed stronger nuclear YAP staining. These observations show that dysregulation of YAP protein level and localization indeed occurs in human HCC. Similar observations were made in prostate cancer tissues ($p=0.004$) (Fig.2.13A, 2.13B). We speculate that YAP activation in cancer tissues is likely due to mutation or dysregulation of the Hippo pathway include YAP itself, and that uncontrolled YAP activation may contribute to cancer development.

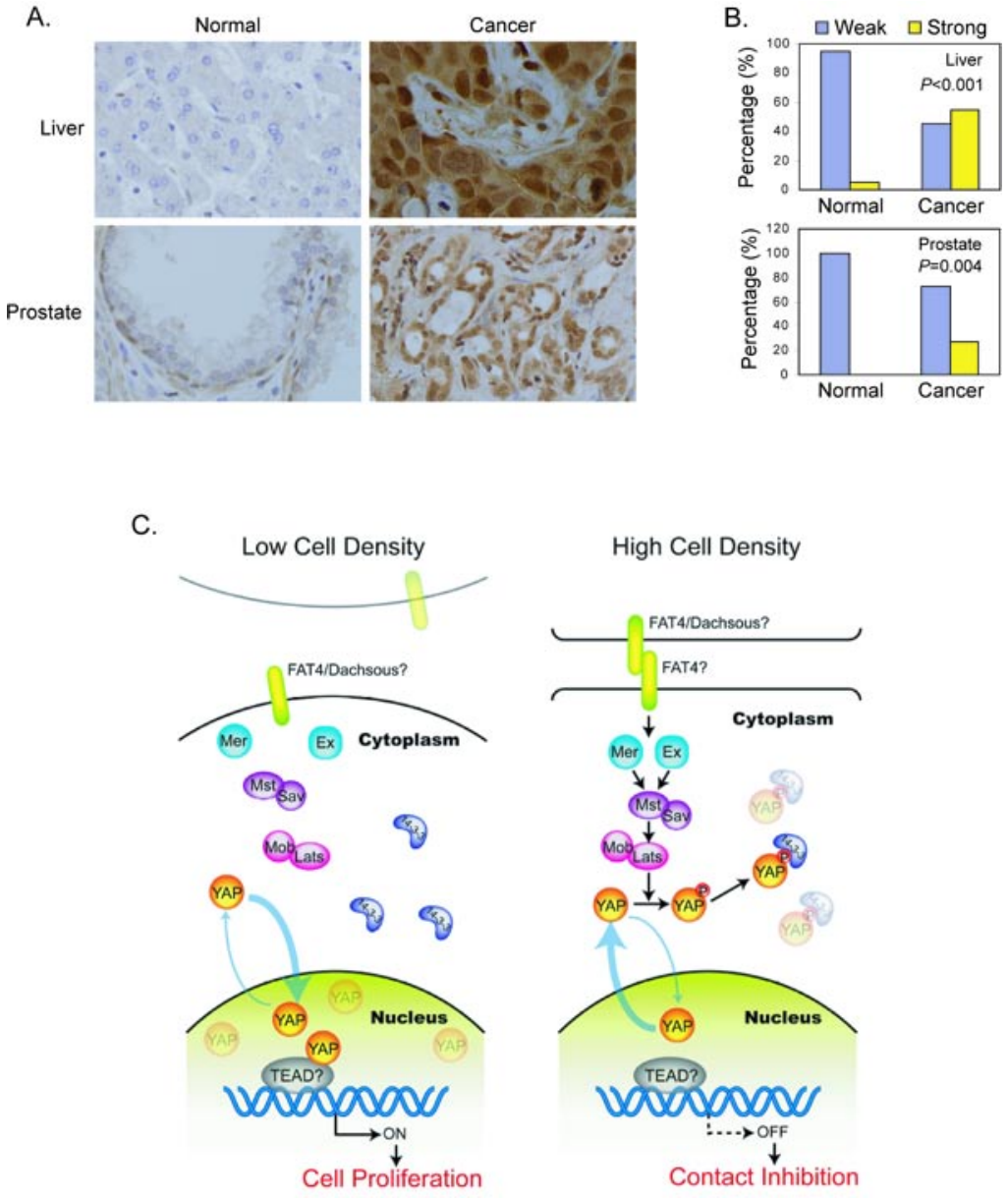


Figure 2.13 Elevated YAP protein and nuclear localization in human cancers.
A. Tissue microarrays of liver and prostate cancer were stained with anti-YAP antibody (brown). Cell nuclei were counterstained with Hematoxylin (blue).
B. Nuclear YAP protein is significantly elevated in human cancers. Samples were scored based on median nuclear staining intensity, ranging from 0 to 6 (0 for negative and 6 for very strong staining). Strong staining was considered a score of 2 or higher for liver and 3 or higher for prostate. *P* values (Fisher exact test) indicate the differences in the proportions of strong YAP staining between cancer and normal specimens.
C. A model for YAP regulation by cell contact via the Hippo pathway.

Discussion

Recent studies have implicated YAP as an oncogene (Overholtzer et al. 2006; Zender et al. 2006). However, neither the precise biological function nor the physiological regulation of YAP is clear. Our study demonstrates that YAP functions downstream of and is inhibited by the Hippo pathway in cell contact inhibition (Fig.2.13C).

It has been previously reported that YAP2 S127 is phosphorylated by Akt in response to growth factor stimulation (Basu et al., 2003). However, we found that phosphorylation of YAP2 S127 is not affected in PDK1 knockout cells, in which the Akt activity is abolished. Furthermore, EGF, insulin, PI3K inhibitors, and Akt overexpression did not affect YAP phosphorylation in our experiments. In contrary, we presented data to show that S127 in YAP2 is directly phosphorylated by Lats. Given the fact that YAP is an oncogene and its activity is inhibited by S127 phosphorylation, the previous model that Akt phosphorylates YAP S127 is inconsistent with the known function of Akt. We conclude that Lats plays a direct role in YAP phosphorylation and inhibition. However, it is still possible that YAP might be phosphorylated by Akt under some physiological or pathological conditions.

YAP in Cell Contact Inhibition

An important observation described in this study is the dramatic translocation of YAP between the nucleus and the cytoplasm in response to cell density status. We propose a model that upon cell-cell contact, certain cell surface receptors (Fat is a possible candidate) are activated via interaction with other surface proteins (such as Dachsous) (Matakatsu and Blair 2004). The activated receptor then stimulates Merlin and Expanded, which in turn stimulate Mst/Sav protein kinase activity. Active Mst/Sav phosphorylates and activates the Lats/Mob complex, which directly phosphorylates YAP on HXRXXS motifs. Phosphorylated YAP then associates with 14-3-3 and is sequestered in the cytoplasm (Fig.2.13C). Remarkably, genetic screens in Drosophila identified three

Yki gain-of-function alleles, and mutation of the corresponding residues in YAP decreases phosphorylation or 14-3-3 binding. These results demonstrate the functional importance and the inhibitory nature of the phosphorylation of YAP-S127 (Yki-S168) by the Hippo pathway. However, additional mechanisms of YAP regulation may also exist, because YAP-5SA can be partially inhibited by Mst and Lats in the reporter assays and the subcellular localization of this mutant still shows partial response to cell density.

Several lines of evidence support the function of YAP in contact inhibition. First, cell density regulates Lats kinase activity and YAP nuclear/cytoplasmic shuttling. Second, scratching of confluent cultured cells induces YAP nuclear localization in cells at the wound edge. Those cells with nuclear YAP also enter the cell cycle. Third, YAP-overexpressing cells fail to exit the cell cycle when confluent and grow to a much higher density. In fact, there is a strong correlation between nuclear YAP protein levels and staining with the proliferation marker Ki67 (data not shown). Fourth, expression of dominant-negative YAP restores contact inhibition in a human cancer cell line bearing deletion of Sav. Fifth, YAP regulates many genes in a manner opposite to high cell density. Furthermore, YAP is inhibited by Merlin, which has been implicated in mediating cell contact inhibition (Lallemand et al. 2003; Okada et al. 2005). Together, our study indicates that YAP plays a critical role in cell contact inhibition and that the Hippo pathway may relay cell contact signals to inactivate YAP, thereby, inducing contact inhibition (Fig.2.13C).

YAP in Tumorigenesis

Our data shows that YAP expression is frequently elevated in human cancers. More than 50% of hepatocellular carcinoma (HCC) examined have increased nuclear YAP protein levels. Prostate cancers also have significant elevation of YAP protein levels and nuclear localization, although at a lower frequency. These data indicate that YAP may play an important role in human tumorigenesis. However, in spite of the high frequency of YAP overexpression we observed, a relatively low incidence (5-15%) of

amplification of the human chromosome 11q22 amplicon has been reported in human tumors (Baldwin et al. 2005; Snijders et al. 2005; Zender et al. 2006). Thus, we speculate that elevation of YAP protein levels in cancer is not entirely due to gene amplification but may instead result from dysregulation of the Hippo pathway. For example, mutation of NF2 should result in inhibition of the Hippo pathway and subsequent activation of YAP by abrogation of inhibitory phosphorylation. We propose that Merlin functions as a tumor suppressor at least in part by inactivating the YAP oncoprotein. It has also been reported that both Sav and Mob are mutated in tumor cell lines (Tapon et al. 2002; Lai et al. 2005). Therefore, mutation or dysregulation of Mst/Sav and Lats/Mob may contribute to uncontrolled YAP activation in human cancers.

Constitutive activation of YAP may cause evasion of contact inhibition, therefore providing a growth advantage for YAP-overexpressing cancer cells. YAP may also be activated in other cancer types. Future studies to elucidate the Hippo pathway and YAP regulation will not only provide new insights into cell growth regulation, especially cell contact inhibition, but will also be valuable in understanding tumorigenesis. Pharmacologic intervention in the Hippo pathway, for example, inhibition of YAP, may be an effective strategy to treat cancers exhibiting YAP activation and/ or overexpression.

Many important questions in the Hippo pathway remain to be addressed. For example, little is known about how Merlin is activated by cell contact. One possibility is CD44, a cell surface receptor implicated in cell contact inhibition, acts upstream of Merlin (Morrison et al. 2001). It is also possible that Fat4 may function upstream of Merlin or Ex1 to initiate the cell contact signaling pathway. Another key question is the mechanism of Mst activation by Merlin. Also elusive is the critical transcription factor(s) mediating the physiological function of YAP. We speculate that TEAD may have a role in YAP function. Interestingly, *Scalloped*, the *Drosophila TEAD* homolog, plays some roles in regulating cell proliferation and apoptosis (Delanoue et al. 2004), suggesting an intriguing possibility of Scalloped as a Yki target transcription factor. The *bantam* microRNA plays a critical role in *Drosophila* to mediate the Hippo pathway signaling, but there is no obvious *bantam* homolog in human genome (Nolo et al. 2006; Thompson

and Cohen 2006). It will be interesting to see if functionally similar microRNA exists in humans to mediate the physiological function of YAP.

In summary, our study demonstrates that inactivation of the YAP oncoprotein may play a critical role in cell contact inhibition. This is at least partially accomplished by the Hippo pathway-dependent phosphorylation that promotes YAP binding to 14-3-3 and cytoplasmic localization. Dysregulation of YAP evades contact inhibition and may contribute to tumorigenesis.

Contents of this chapter were published in the following paper: Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.C., and Guan, K.L. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* **21**(21): 2747-2761.

Bibliography

Abercrombie, M. 1979. Contact inhibition and malignancy. *Nature* **281**(5729): 259-262.

Baldwin, C., Garnis, C., Zhang, L., Rosin, M.P., and Lam, W.L. 2005. Multiple microalterations detected at high frequency in oral cancer. *Cancer Res* **65**(17): 7561-7567.

Basu, S., Totty, N.F., Irwin, M.S., Sudol, M., and Downward, J. 2003. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Mol Cell* **11**(1): 11-23.

Bennett, F.C. and Harvey, K.F. 2006. Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. *Curr Biol* **16**(21): 2101-2110.

Cho, E., Feng, Y., Rauskolb, C., Maitra, S., Fehon, R., and Irvine, K.D. 2006. Delineation of a Fat tumor suppressor pathway. *Nat Genet* **38**(10): 1142-1150.

Conlon, I. and Raff, M. 1999. Size control in animal development. *Cell* **96**(2): 235-244.

Delanoue, R., Legent, K., Godefroy, N., Flagiello, D., Dutriaux, A., Vaudin, P., Becker, J.L., and Silber, J. 2004. The Drosophila wing differentiation factor vestigial-scalloped is required for cell proliferation and cell survival at the dorso-ventral boundary of the wing imaginal disc. *Cell Death Differ* **11**(1): 110-122.

Eagle, H. and Levine, E.M. 1967. Growth regulatory effects of cellular interaction. *Nature* **213**(5081): 1102-1106.

Edgar, B.A. 2006. From cell structure to transcription: Hippo forges a new path. *Cell* **124**(2): 267-273.

Halbleib, J.M. and Nelson, W.J. 2006. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* **20**(23): 3199-3214.

Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., and Halder, G. 2006. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. *Nat Cell Biol* **8**(1): 27-36.

Hanahan, D. and Weinberg, R.A. 2000. The hallmarks of cancer. *Cell* **100**(1): 57-70.

Hariharan, I.K. 2006. Growth regulation: a beginning for the hippo pathway. *Curr Biol* **16**(24): R1037-1039.

Harvey, K. and Tapon, N. 2007. The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. *Nat Rev Cancer* **7**(3): 182-191.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. 2003. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**(4): 457-467.

Hergovich, A., Stegert, M.R., Schmitz, D., and Hemmings, B.A. 2006. NDR kinases regulate essential cell processes from yeast to humans. *Nat Rev Mol Cell Biol* **7**(4): 253-264.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**(3): 421-434.

Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P.R., Bellen, H.J., and Halder, G. 2002. Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. *Development* **129**(24): 5719-5730.

Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., and Li, Y. 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120**(5): 675-685.

Lallemand, D., Curto, M., Saotome, I., Giovannini, M., and McClatchey, A.I. 2003. NF2 deficiency promotes tumorigenesis and metastasis by destabilizing adherens junctions. *Genes Dev* **17**(9): 1090-1100.

Mah, A.S., Elia, A.E., Devgan, G., Ptacek, J., Schutkowski, M., Snyder, M., Yaffe, M.B., and Deshaies, R.J. 2005. Substrate specificity analysis of protein kinase complex Dbf2-Mob1 by peptide library and proteome array screening. *BMC Biochem* **6**: 22.

Matakatsu, H. and Blair, S.S. 2004. Interactions between Fat and Dachsous and the regulation of planar cell polarity in the Drosophila wing. *Development* **131**(15): 3785-3794.

McClatchey, A.I. and Giovannini, M. 2005. Membrane organization and tumorigenesis--the NF2 tumor suppressor, Merlin. *Genes Dev* **19**(19): 2265-2277.

Morrison, H., Sherman, L.S., Legg, J., Banine, F., Isacke, C., Haipek, C.A., Gutmann, D.H., Ponta, H., and Herrlich, P. 2001. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* **15**(8): 968-980.

Muslin, A.J. and Xing, H. 2000. 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal* **12**(11-12): 703-709.

Nolo, R., Morrison, C.M., Tao, C., Zhang, X., and Halder, G. 2006. The bantam microRNA is a target of the hippo tumor-suppressor pathway. *Curr Biol* **16**(19): 1895-1904.

Okada, T., Lopez-Lago, M., and Giancotti, F.G. 2005. Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. *J Cell Biol* **171**(2): 361-371.

Okada, T., You, L., and Giancotti, F.G. 2007. Shedding light on Merlin's wizardry. *Trends Cell Biol* **17**(5): 222-229.

Overholtzer, M., Zhang, J., Smolen, G.A., Muir, B., Li, W., Sgroi, D.C., Deng, C.X., Brugge, J.S., and Haber, D.A. 2006. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc Natl Acad Sci U S A* **103**(33): 12405-12410.

Pan, D. 2007. Hippo signaling in organ size control. *Genes Dev* **21**(8): 886-897.

Pantalacci, S., Tapon, N., and Leopold, P. 2003. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat Cell Biol* **5**(10): 921-927.

Rong, R., Surace, E.I., Haipek, C.A., Gutmann, D.H., and Ye, K. 2004. Serine 518 phosphorylation modulates merlin intramolecular association and binding to critical effectors important for NF2 growth suppression. *Oncogene* **23**(52): 8447-8454.

Silva, E., Tsatskis, Y., Gardano, L., Tapon, N., and McNeill, H. 2006. The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. *Curr Biol* **16**(21): 2081-2089.

Snijders, A.M., Schmidt, B.L., Fridlyand, J., Dekker, N., Pinkel, D., Jordan, R.C., and Albertson, D.G. 2005. Rare amplicons implicate frequent deregulation of cell fate specification pathways in oral squamous cell carcinoma. *Oncogene* **24**(26): 4232-4242.

St John, M.A., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M.L., Brownstein, D.G., Parlow, A.F., McGrath, J., and Xu, T. 1999. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat Genet* **21**(2): 182-186.

Takahashi, Y., Miyoshi, Y., Takahata, C., Irahara, N., Taguchi, T., Tamaki, Y., and Noguchi, S. 2005. Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. *Clin Cancer Res* **11**(4): 1380-1385.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. 2002. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**(4): 467-478.

Thompson, B.J. and Cohen, S.M. 2006. The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. *Cell* **126**(4): 767-774.

Tyler, D.M. and Baker, N.E. 2007. Expanded and fat regulate growth and differentiation in the Drosophila eye through multiple signaling pathways. *Dev Biol* **305**(1): 187-201.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat Cell Biol* **5**(10): 914-920.

Vassilev, A., Kaneko, K.J., Shu, H., Zhao, Y., and DePamphilis, M.L. 2001. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev* **15**(10): 1229-1241.

Wei, X., Shimizu, T., and Lai, Z.C. 2007. Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. *Embo J* **26**(7): 1772-1781.

Willecke, M., Hamaratoglu, F., Kango-Singh, M., Udan, R., Chen, C.L., Tao, C., Zhang, X., and Halder, G. 2006. The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Curr Biol* **16**(21): 2090-2100.

Williams, M.R., Arthur, J.S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., and Alessi, D.R. 2000. The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr Biol* **10**(8): 439-448.

Wu, S., Huang, J., Dong, J., and Pan, D. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**(4): 445-456.

Yagi, R., Chen, L.F., Shigesada, K., Murakami, Y., and Ito, Y. 1999. A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. *Embo J* **18**(9): 2551-2562.

Yin, F. and Pan, D. 2007. Fat flies expanded the hippo pathway: a matter of size control. *Sci STKE* **2007**(380): pe12.

Zender, L., Spector, M.S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S.T., Luk, J.M., Wigler, M., Hannon, G.J., Mu, D., Lucito, R., Powers, S., and Lowe, S.W. 2006. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* **125**(7): 1253-1267.

CHAPTER 3

TEAD MEDIATES YAP-DEPENDENT GENE INDUCTION AND GROWTH CONTROL

Abstract

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 are 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.

Introduction

Recent genetic studies in *Drosophila* have identified a novel tumor suppressor pathway, the Hippo pathway (Harvey et al. 2003; Wu et al. 2003; Lai et al. 2005; Edgar 2006; Hariharan and Bilder 2006; Harvey and Tapon 2007). Genetic experiments demonstrated that the Yki transcription coactivator is inhibited by the Hippo pathway (Huang et al. 2005). Consistently, biochemical studies showed that Yki is directly phosphorylated and inhibited by the Wts protein kinase, which is phosphorylated and

activated by the Hippo (Hpo) protein kinase (Dong et al. 2007). Yki induces expression of genes like cyclin E and Diap1, therefore promotes proliferation and inhibits apoptosis (Udan et al. 2003; Huang et al. 2005). However, Yki does not have DNA binding domain, therefore, must interact with a DNA binding transcription factor(s) to regulate gene expression. Scalloped (Sd), a transcription factor in *Drosophila*, has recently been reported to act downstream of Yki (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008).

Components of the Hippo pathway are highly conserved, and recent studies from us and other groups have demonstrated the function of the Hippo pathway in mammalian cell growth (Hao et al. 2007; Zhao et al. 2007). YAP, the human homolog of Yki, is phosphorylated by the Lats tumor suppressor, which is a homolog of the *Drosophila* Wts. Phosphorylation of YAP by Lats results in cytoplasmic translocation, therefore, inactivation of YAP. This mechanism of YAP regulation is involved in cell contact inhibition and tissue growth control (Zhao et al. 2007).

The importance of the Hippo pathway in human cancer was gradually uncovered. Mutation of the Hippo pathway components, such as the NF2 tumor suppressor, is known to contribute to human tumorigenesis (McClatchey and Giovannini 2005). More importantly, YAP is the candidate oncogene in the human chromosome 11q22 amplicon, which is evident in several human cancers (Overholtzer et al. 2006; Zender et al. 2006). YAP overexpression stimulates proliferation and increases saturation cell density in monolayer culture of NIH-3T3 cells (Zhao et al. 2007). Furthermore, YAP overexpression in MCF10A cells induces epithelial-mesenchymal transition (EMT), which is a hallmark of tumorigenic transformation (Overholtzer et al. 2006). Moreover, elevated YAP protein levels and increased nuclear localization have been observed in multiple human cancer tissues (Zhao et al. 2007). Interestingly, YAP overexpression causes a dramatic increase in liver size and eventually leads to tumor growth (Camargo et al. 2007; Dong et al. 2007). These observations have established 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 RNA interference, we further showed that TEAD is required for YAP to stimulate gene expression, cell growth, anchorage-independent growth, and EMT. We have 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 of YAP.

Materials and Methods

Antibodies, Plasmids, and Materials

Anti-YAP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TEAD1, E-cadherin, N-Cadherin, gamma-Catenin, Fibronectin, Hsp90, Alexa Fluor 488 mouse anti-human Ki67, and FITC mouse anti-E-Cadherin were obtained from BD Biosciences (San Jose, CA). Anti-α-Tubulin and anti-Flag antibodies were obtained from Sigma (St. Louis, MO). Anti-Myc antibodies were obtained from Covance (Philadelphia, PA). Rhodamine phalloidin was obtained from Invitrogen (Carlsbad, CA). Horseradish peroxidase conjugated secondary antibodies were obtained from Amersham (Buckinghamshire, UK).

The pCMV-Flag-YAP and the pM-ErbB4-CTFΔK constructs were kindly provided by Dr. Marius Sudol, and YAP was subcloned into the pQCXIH-Myc retrovirus vector. The YAP 5SA and S94A mutants were generated by site-directed mutagenesis. The RUNX2 and 6×OSE2-luc reporter were from Dr. Hongjiao Ouyang. CTGF 250bp promoter was cloned from human genomic DNA into the pGL3-Basic vector. The ΔTB

mutants were generated by mutating the GGAATG sites to GGGCGG. TEAD1 was cloned from a HeLa cDNA library into the pCMX-Gal4 and pQCXIH vectors. TEAD1-YAP-S94A fusion was generated by cloning YAP-S94A into pQCXIH-TEAD1 following and in frame with the TEAD1 coding region. TEAD4 was cloned from a HeLa cDNA library into the PRK5-Myc vector. The TEAD1-ΔC construct was generated by truncating the C terminal of TEAD1 after amino acid 289. And the TEAD1-ΔC-AD was generated by further fusion with the YAP C-terminal activation domain after amino acid 290.The 5×UAS-luciferase reporter was described before (Zhao et al. 2007) and the Gal4-TEAD2, 3, and 4 constructs were isolated from a GAL4-TF library (Dr. Jiandie Lin).

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 (Invitrogen) and 50μg/ml penicillin/streptomycin (P/S). MCF10A cells were cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20ng/ml EGF, 0.5μg/ml hydrocortisone, 10μg/ml insulin, 100ng/ml cholera toxin, and 50μg/ml P/S. Transfection with lipofectamine was performed according to the manufacturer's instructions.

To generate wild type or mutant YAP expressing stable cells, retrovirus infection was performed by transfecting 293 Phoenix retrovirus packaging cells with empty vector or pQCXIH-YAP constructs. 48 hours after transfection, retroviral supernatant was supplemented with 5μg/ml polybrene, filtered through a 0.45μm filter, and used to infect MCF10A or NIH-3T3 cells. 36 hours after infection, cells were selected with 200μg/ml hygromycin (Roche) in culture medium.

Lentiviral shRNA Cloning, Production, and Infection

To generate YAP, TEAD1/3/4, or CTGF knockdown cells, oligonucleotides were cloned into pLKO.1 with the AgeI/EcoRI sites (Moffat et al. 2006). TEAD1/3/4 shRNAs were designed in a region identical in TEAD1, 3, and 4. The sequences of the oligonucleotides are as follows:

YAP #1-sense:

5’CCGGCTGGTCAGAGATACTTCTTAACTCGAGTTAAGAAGTATCTCTGACCA GTTTTTC

YAP #1-antisense:

5’AATTGAAAAACTGGTCAGAGATACTTCTTAACTCGAGTTAAGAAGTATCTC TGA CCAG

YAP #2-sense:

5’CCGGAAGCTTTGAGTTCTGACATCCCTCGAGGGATGTCAGAACTCAAAGCT TTTTTTC

YAP #2-antisense:

5’AATTGAAAAAAAGCTTTGAGTTCTGACATCCCTCGAGGGATGTCAGAACTC AAAGCTT

TEAD1/3/4 #1-sense:

5’CCGGATGATCAACTTCATCCACAAGCTCGAGCTTGTGGATGAAGTTGATCA TTTTTTC

TEAD1/3/4 #1-antisense:

5’AATTGAAAAAATGATCAACTTCATCCACAAGCTCGAGCTTGTGGATGAAGT TGATCAT

TEAD1/3/4 #2-sense:

5’CCGGGATCAACTTCATCCACAAGCTCTCGAGAGCTTGTGGATGAAGTTGATCTTTTTC

TEAD1/3/4 #2-antisense:

5’AATTGAAAAAGATCAACTTCATCCACAAGCTCTCGAGAGCTTGTGGATGAAGTTGATC

CTGF #1-sense:

5’CCGGAAATCTCCAAGCCTATCAAGTCTCGAGACTTGATAGGCTTGGAGATTTTTTTTC

CTGF #1-antisense:

5’AATTGAAAAAAAATCTCCAAGCCTATCAAGTCTCGAGACTTGATAGGCTTGGAGATTT

CTGF #2-sense:

5’CCGGCTGCACCAGCATGAAGACATACTCGAGTATGTCTTCATGCTGGTGCAGTTTTTC

CTGF #2-antisense:

5’AATTGAAAAACTGCACCAGCATGAAGACATACTCGAGTATGTCTTCATGCTGGTGCAG

Plasmids were propagated in and purified from Stbl2 competent cells (Invitrogen). The infection process was similar to that of retroviral infection except that the lentiviral packaging plasmids psPAX2 and pMD2.G were co-transfected into HEK293-T cells for virus production. Cells were selected in 5μg/ml puromycin in culture medium.

Luciferase Assay and Gal4-TF Library Screen

For the luciferase reporter assay, HEK293-T cells were seeded in 12 well plates. Luciferase reporter, CMV-β-gal, and indicated plasmids were co-transfected. 36 hours after transfection, cells were lysed and luciferase activity was assayed using the enhanced luciferase assay kit obtained from BD Biosciences (San Jose, CA) following the manufacturer's instructions. All luciferase activities were normalized to β-galactosidase activity. The Gal4-TF library is in 96-well format with a distinct transcript factor in each well. The Gal4-fusion transcription factors from each plate was transfected into two 96-well plates seeded with HEK293-T cells with the 5×UAS-luciferase reporter and with or without YAP. Luciferase assay was carried out as stated above and the reading from the well with YAP was divided by the reading of the respective well without YAP to get the activation fold. The screen was done in duplicate.

Soft Agar Colony Formation Assay

Cells (5×10^3) were added to 1.5ml growth medium with 0.4% agarose and layered onto 2ml of 0.5% agarose beds in 6-well plates. Cells were fed with 2ml growth medium every week for 3 weeks, after which colonies were fixed with 10% acetic acid/ 10% methanol for 10 minutes followed by staining with 0.005% Crystal Violet for 1 hour. Pictures were taken and colonies were counted under a dissecting microscope.

Chromatin Immunoprecipitation and ChIP-on-chip

ChIP-on-chip and Genome-wide location analysis were performed as previously described (Yu et al. 2007). Briefly, cells were cross-linked, lysed, and sonicated to generate DNA fragments with an average size of 0.5 kb. ChIP was performed using 5ug of antibodies against YAP, TEAD1, AR or control IgG. ChIP-enriched DNA, along with input whole lysate DNA, were subjected to a ligation-mediated PCR step to generate enough DNA materials, which were then labeled with fluorescent dyes and hybridized to a promoter microarray 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, less than 0.001.

Three Dimensional Culture of MCF10A Cells

The 3D culture of MCF10A cells was done as described (Debnath et al. 2003). 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 5000 cells/ well in assay medium containing 2% matrigel and 5 ng/ml EGF. Cells were cultured in a 5% CO_2 humidified incubator at 37°C. The medium was replaced every 4 days.

Drosophila Genetics

For *in vivo* functional analysis of YAP/ Yki, full-length cDNAs of YAP or *yki* were cloned into a transformation vector *pUAST* (Brand and Perrimon 1993). Multiple transgenic fly lines were generated for each of the following DNA constructs: *pUAS-Flag-YAP*$^{S94A/S127A}$ (15 lines) and *pUAS-yki*^{S97A}*-V5* (6 lines). Both *pUAS-Flag-YAP*^{S127A} and *pUAS-yki-V5* were previously published (Zhao et al. 2007). *GMR-Gal4* drives eye-specific expression of *UAS* transgenes. Scanning electron microscopy (SEM) was used to reveal adult eye phenotypes. Immunofluorescent staining of mid-pupal eye discs was done with mouse anti-Discs large (Dlg) (DSHB, 1:300) as primary antibody and Alexa Fluor 488 (Molecular Probes, 1:300) as secondary antibody. For clonal overexpression analysis of Yki and YAP, corresponding UAS transgenic flies were crossed with *w, hsFLP; act>w*$^{+}$*>Gal4; UAS-GFP/TM6B* and progenies were raised at 20°C. Four days later, the flies were heat-treated at 31°C for one hour and then left at 20°C for another three days. Late third instar larvae were dissected and wing imaginal discs were fixed in

8% paraformaldehyde-lysine-phosphate (PLP) buffer for 45 minutes at 4°C. GFP signal was observed by confocal microscopy.

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 1,100, JDL, unpublished) was individually co-transfected with a 5×UAS-luciferase reporter, which is driven by 5 Gal4 binding elements, in the presence or absence of YAP co-transfection. 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.3.1A). 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.3.1A). 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 serine 94 to alanine (S94A) mutant was defective in TEAD4 activation (Fig.3.1B) as well as other TEADs activation (data not shown). However, the YAP-S94A retains full potential to activate RUNX2 (Fig.3.1B) 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.3.1C) 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. Our data showed that YAP-5SA caused a stronger induction of YAP inducible genes than the wild type YAP (Fig.3.1D). Interestingly, YAP-S94A was severely compromised in gene regulation (both induction and repression) (Fig.3.1D). We have previously reported 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 (Fig.3.2A). The majority of genes that are affected by YAP expression are similarly regulated (either up or down) in both NIH-3T3 and MCF10A cells, while a subset of genes being oppositely regulated in NIH-3T3 and MCF10A cells.

Among the confirmed YAP inducible genes in MCF10A were CTGF and ITGB2 (integrin beta 2). They were strongly induced by YAP-5SA but not by YAP-S94A (Fig.3.2B). Furthermore, co-expression 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 (Fig.3.2B). 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, 3, and 4. Indeed, these shRNAs were able to knockdown TEAD1, 3, and 4 concurrently but not TEAD2 (Fig.3.2C). Nevertheless, these TEAD1/3/4 shRNAs strongly blocked the induction of CTGF and ITGB2 by YAP-5SA expression (Fig.3.1E). 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 ChIP-on-chip experiments. Interestingly, our results demonstrated that YAP and TEAD1 co-occupy over 80% of the

promoters pulled down by either of them (Fig.3.1F). 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 3.1 TEAD is required for YAP induced gene expression.

A. YAP potently activates TEAD family transcription factors. Indicated Gal4-fused transcription factors were co-transfected with a 5×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 one. Flag-YAP western blot shows that YAP expression level was not decreased by ErbB4.

B. YAP-S94A cannot activate TEAD4. Indicated plasmids were co-transfected with a 5×UAS-luciferase reporter for Gal4-TEAD4 or a 6×OSE2-luciferase reporter for RUNX2 into 293T cells. Luciferase activity was measured and normalized to co-transfected β-galactosidase.

C. Serine 94 of YAP is required for its interaction with TEAD4. 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. 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 5-fold or down-regulated more than 4-fold by YAP-WT overexpression. Cluster analysis was done with Eisen Lab Cluster software using average linkage clustering. The same data sets were drawn into boxplots using the R program (Right panel). Red and green indicate up-regulated and down-regulated genes, respectively.

E. TEAD is required for YAP-induced expression of CTGF and ITGB2. 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. C stands for scramble shRNA control, #1 and #2 stand for two different shRNAs targeting TEAD1/3/4.

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.

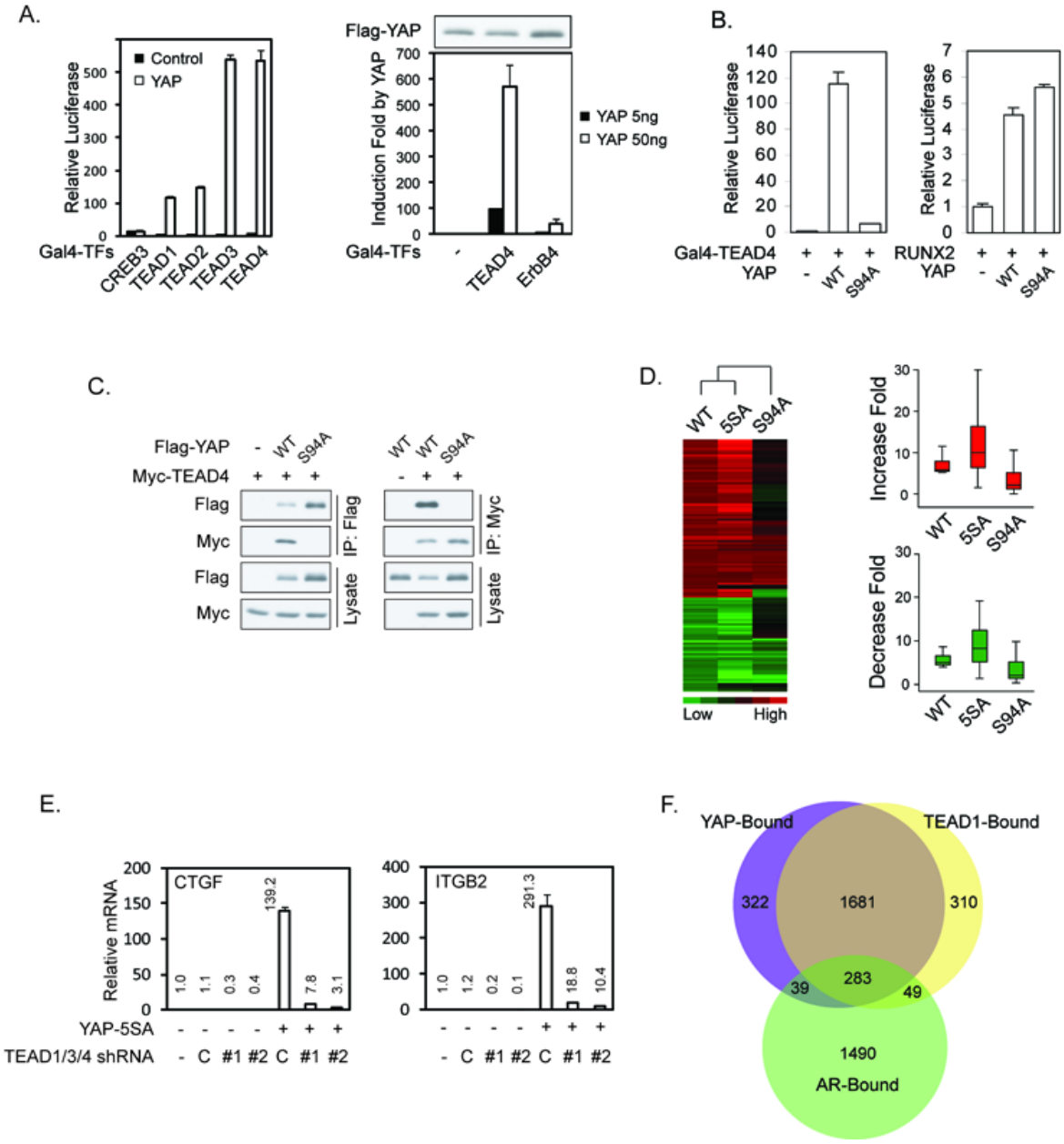
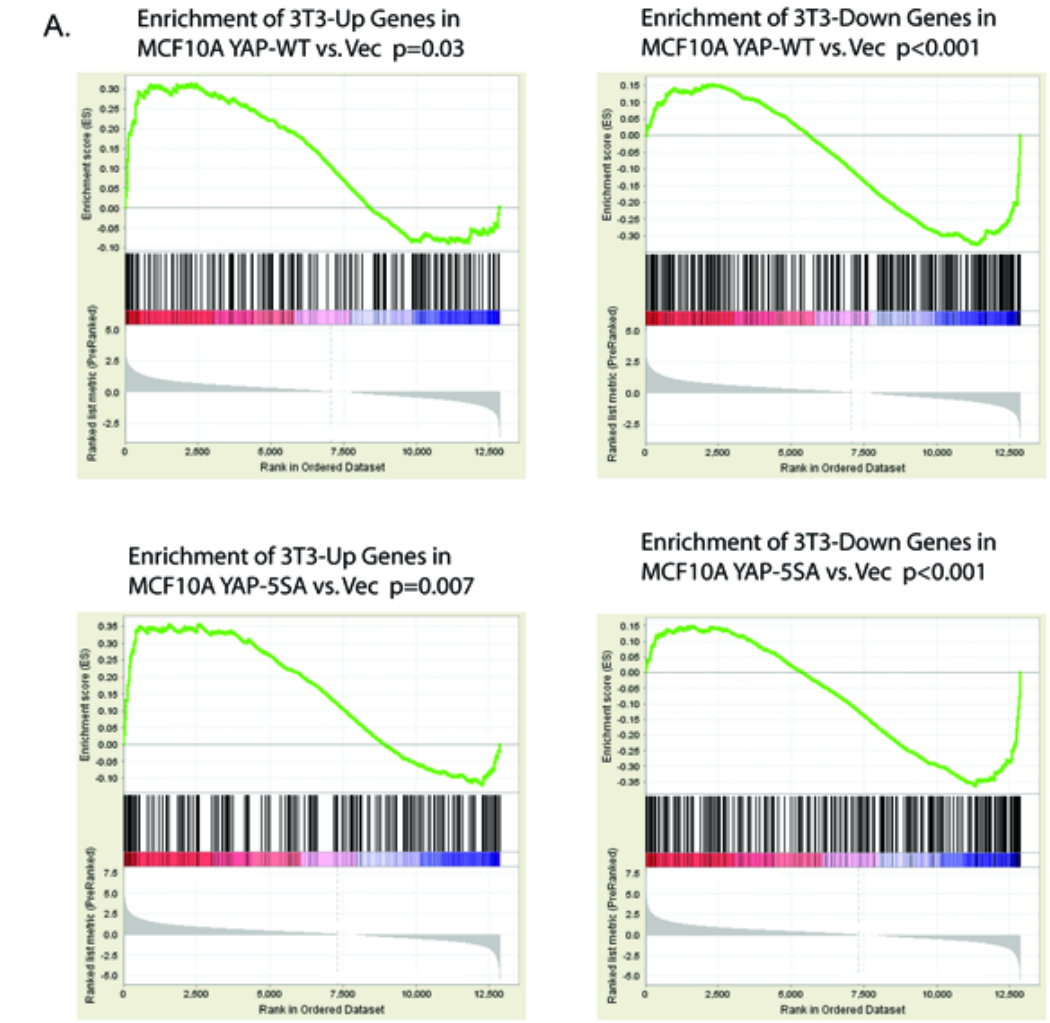

A.
Relative Luciferase
Control
YAP
Gal4-TFs
CREB3
TEAD1
TEAD2
TEAD3
TEAD4
Flag-YAP
Induction Fold by YAP
YAP 5ng
YAP 50ng
Gal4-TFs
TEAD4
ErbB4
B.
Relative Luciferase
Gal4-TEAD4
YAP
WT
S94A
RUNX2
C.
Flag-YAP
Myc-TEAD4
Flag
Myc
IP: Flag
IP: Myc
Lysate
D.
WT
5SA
S94A
Low
High
Increase Fold
Decrease Fold
E.
Relative mRNA
CTGF
ITGB2
YAP-5SA
TEAD1/3/4 shRNA
C
#1
#2
F.
YAP-Bound
TEAD1-Bound
AR-Bound
322
1681
310
39
283
49
1490

Figure 3.2 Supplemental to TEAD is required for YAP induced gene expression.
A. Genes regulated by YAP-WT in NIH-3T3 cells significantly overlapped with genes regulated by YAP-WT or YAP-5SA in MCF10A cells. Genes up-regulated or repressed by YAP in NIH-3T3 cells are determined by a two-fold change of gene expression by YAP-WT overexpression and then cross-linked to human genome based on NetAffy annotation file (Affymetrix) for the corresponding homolog genes. Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005) was then used to examine the similarity between genes regulated by YAP in NIH-3T3 cells and MCF10A cells, which were pre-sorted by fold changes. Four independent analyses were performed, as presented by the four panels, to examine whether genes up/down-regulated by YAP in NIH-3T3 cells are enriched in genes up/down-regulated in MCF10A by YAP-WT or YAP-5SA overexpression relative to vector control. The p value in each panel indicates the statistical significance of the enrichment of up/down-regulated genes in NIH-3T3 cells in corresponding gene set from MCF10A cells. The green curve on the top of each panel represents the accumulated enrichment score (represented by the density of the vertical black bars) of NIH-3T3 gene set along the pre-ranked genes (gray shaded area at the bottom of each panel) of the MCF10A gene set with the left end for up-regulated (red bars) and the right end for down-regulated (blue bars) genes.
B. Induction of CTGF and ITGB2 by YAP requires TEAD. MCF10A cells stably expressing wild type and various mutant YAP were generated. TEAD1-ΔC indicates the C-terminal YAP-interacting domain deleted form of TEAD1. CTGF and ITGB2 mRNA levels were measured by quantitative RT-PCR.
C. Knockdown of TEAD1/3/4 by shRNAs. The TEAD1/3/4 shRNA #1 and #2 effectively decreased TEAD1, 3, and 4 but not TEAD2 expression in MCF10A cells.


A.
Enrichment of 3T3-Up Genes in MCF10A YAP-WT vs. Vec p=0.03
Enrichment of 3T3-Down Genes in MCF10A YAP-WT vs. Vec p<0.001
Enrichment of 3T3-Up Genes in MCF10A YAP-5SA vs. Vec p=0.007
Enrichment of 3T3-Down Genes in MCF10A YAP-5SA vs. Vec p<0.001
Enrichment score (ES)
Ranked list metric (PreRanked)
Rank in Ordered Dataset
B.
CTGF
ITGB2
Relative mRNA
1 2 3 4 5
1 Vec
2 YAP-WT
3 YAP-S94A
4 YAP-5SA
5 YAP-5SA + TEAD1-ΔC
C.
TEAD1
TEAD2
TEAD3
TEAD4
Relative mRNA
shRNA
Scramble
TEAD1/3/4 #1
TEAD1/3/4 #2

TEAD Binding Is Required for YAP-induced Cell Growth and EMT

We have 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.3.3A). Furthermore, in MCF10A cells, wild type YAP induced cell proliferation even when cells reached confluent while the YAP-S94A mutant was largely inactive as determined by the staining of proliferation marker Ki67 (Fig.3.4A). To confirm that the loss of growth-promoting activity in YAP-S94A is due to the loss of its interaction with TEAD, we generated 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.3.3A). Furthermore, the TEAD1-YAP-S94A fusion also rescued the expression of Ctgf and Inhba, two YAP target genes, in NIH-3T3 cells (Fig.3.3B). 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 3 dimensional culture compared with vector control. Importantly, this effect was largely reduced if S94A mutation was introduced into YAP-5SA (Fig.3.3C). 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.3.3C). 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.3.3C). As another hallmark of EMT, YAP-5SA-expressing cells also displayed disorganized adheron junctions, as shown by the loss of cell-cell junction localized E-cadherin, and the switch from cortical actin to stress fibers (Fig.3.3D). 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.3.3E). 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 knockdown 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.3.3F, 3.4B). Knockdown of TEAD1/3/4 also significantly shrunk 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 (Fig.3.4C), and is located within the YAP binding domain (Fig. 3.4D). Interestingly, mutation of this tyrosine residue in TEADs abolished their interaction with and their activation by YAP (Fig. 3.4E, F, 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.3.3G, 3.4H). Similarly, almost no colony was formed if TEAD1/3/4 were down-regulated in the YAP-5SA expressing cells (Fig.3.3G, 3.4H). These data indicates the requirement of at least one of TEAD1/3/4 for the YAP-induced anchorage-independent growth. Together, the above observations support a model that TEAD is essential for the function of YAP in cell-proliferation, EMT, and oncogenic transformation.

Figure 3.3 TEAD is required for YAP activity in growth promotion and EMT.
A. YAP-S94A is defective in promoting cell growth. 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. 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 (right panel). The expression of Ctgf and Inhba, two YAP target genes in NIH-3T3 cells, were 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 days before pictures were taken.
D. YAP-5SA-S94A is defective in reducing membrane E-cadherin and cortical actin. 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 panel C.
G. TEAD1/3/4 shRNAs blocked YAP induced anchorage-independent growth in soft agar. Indicated MCF10A stable cells were plated in soft agar and allowed to grow for 3 weeks, after which colonies were stained with crystal violet and counted.

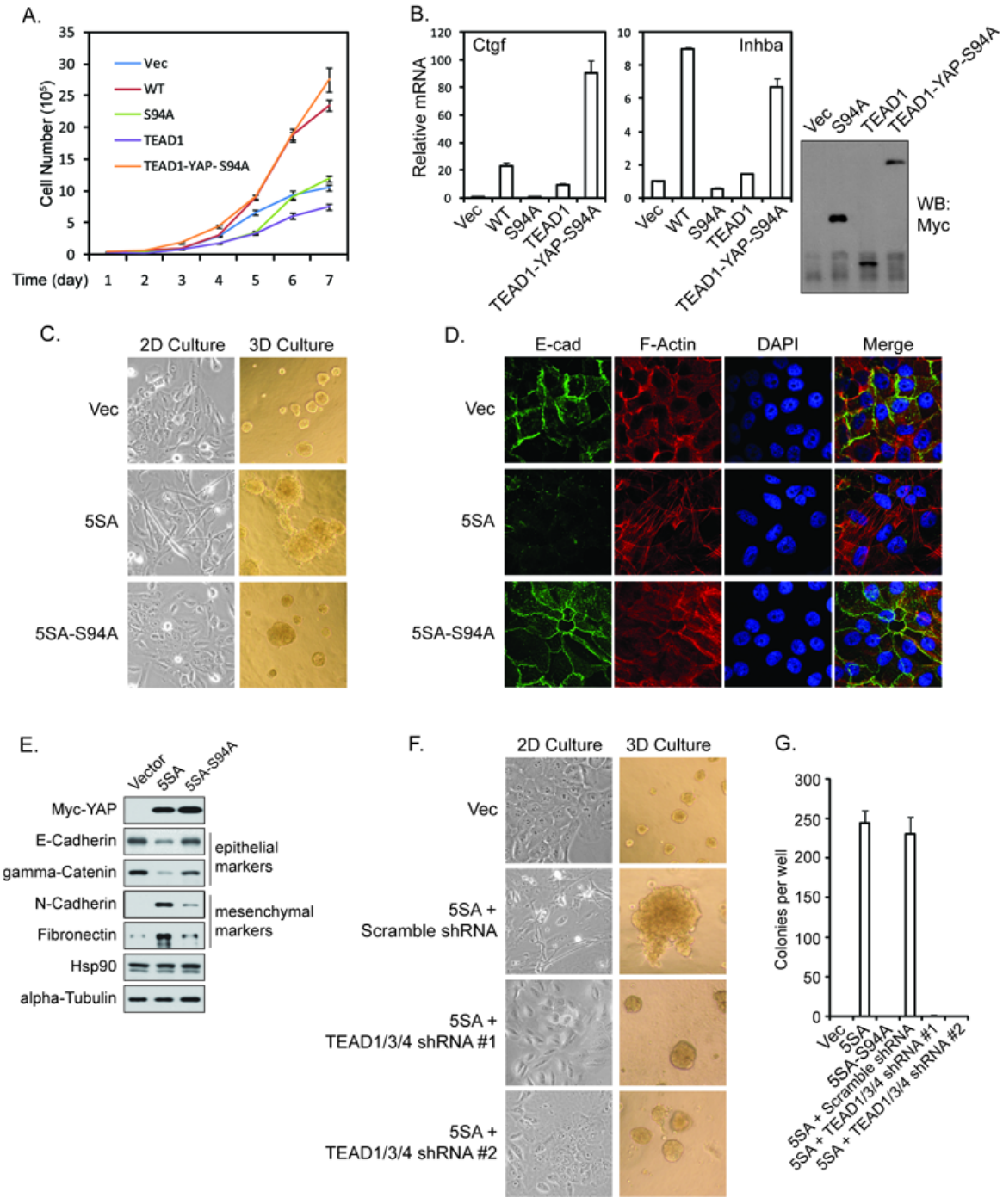
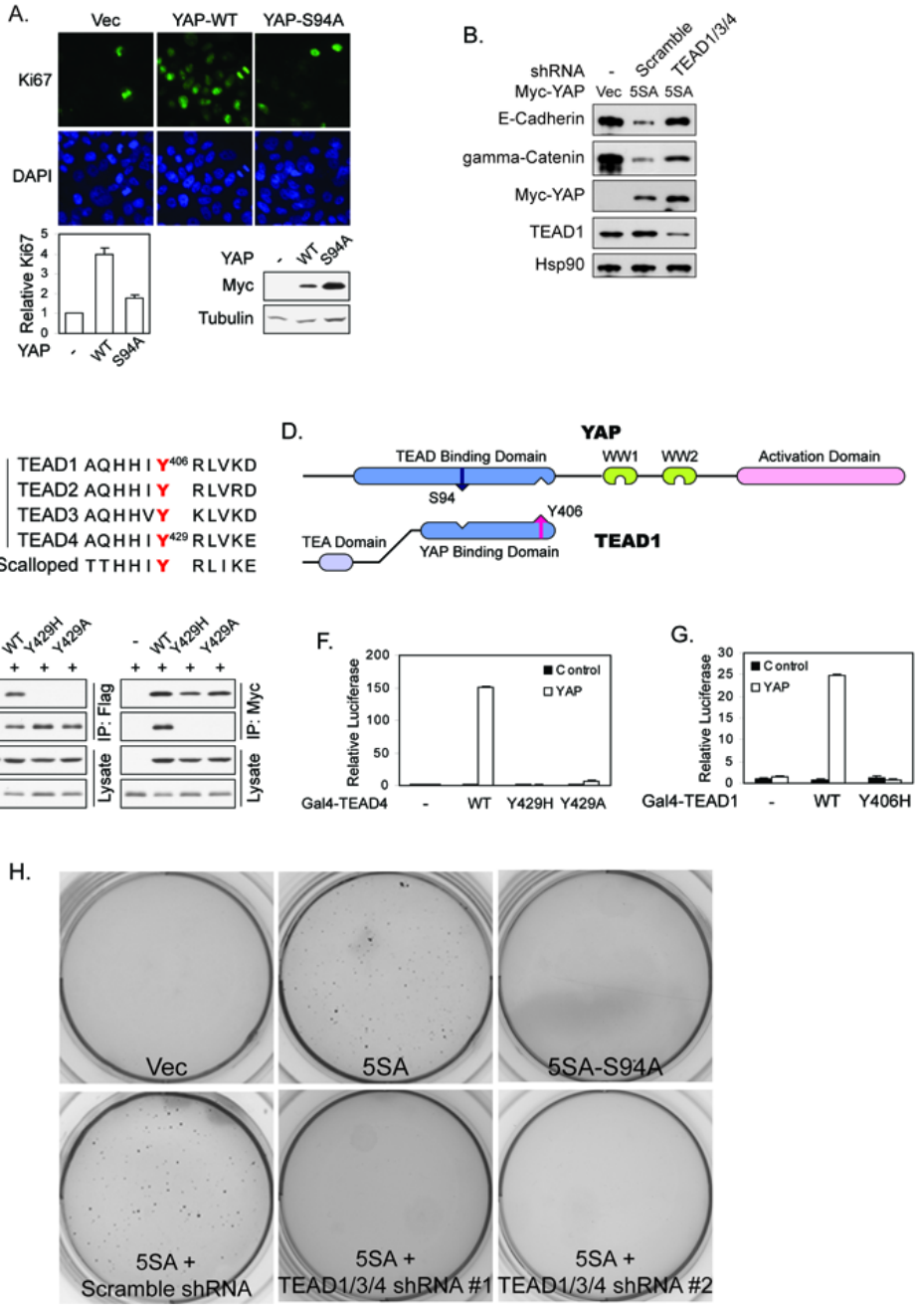

A.
Cell Number (10^5)
Time (day)
Vec
WT
S94A
TEAD1
TEAD1-YAP-S94A
B.
Relative mRNA
Ctgf
Inhba
Vec
WT
S94A
TEAD1
TEAD1-YAP-S94A
WB: Myc
C.
2D Culture
3D Culture
Vec
5SA
5SA-S94A
D.
E-cad
F-Actin
DAPI
Merge
Vec
5SA
5SA-S94A
E.
Vector
5SA
5SA-S94A
Myc-YAP
E-Cadherin
gamma-Catenin
N-Cadherin
Fibronectin
Hsp90
alpha-Tubulin
epithelial markers
mesenchymal markers
F.
2D Culture
3D Culture
Vec
5SA + Scramble shRNA
5SA + TEAD1/3/4 shRNA #1
5SA + TEAD1/3/4 shRNA #2
G.
Colonies per well
Vec
5SA
5SA-S94A
5SA + Scramble shRNA
5SA + TEAD1/3/4 shRNA #1
5SA + TEAD1/3/4 shRNA #2

Figure 3.4 Supplemental to TEAD is required for YAP activity in growth promotion and EMT.

A. YAP-S94A is deficient in inducing proliferation in confluent cells. Stable MCF10A cells infected with indicated plasmids were cultured to confluence. The proliferation marker Ki67 was stained and quantified after Typhoon Imager scanning. The expression of YAP and YAP-S94A was assayed by Western blot.
B. Knockdown of TEAD1/3/4 rescued the reduction of epithelial markers by YAP-5SA expression. MCF10A cells stably expressing YAP-5SA were infected with scramble or TEAD1/3/4 shRNA lentiviruses. Western blotting was performed with epithelial markers E-Cadherin and gamma-Catenin antibodies. Knockdown of TEAD1 was indicated by TEAD1 Western blot.
C. The TEAD1 Y406 mutated in Sveinsson's chorioretinal atrophy is conserved. Alignment of human TEAD family transcription factors and *D. melanogaster* Scalloped protein around the Y406 (TEAD1) residue.
D. Schematic presentation of YAP and TEAD structure. The TEAD binding essential S94 in YAP and the YAP binding essential Y406 in TEAD1 are shown.
E. Y429 in TEAD4 is essential for its interaction with YAP. Flag-YAP and Myc-TEAD4 were co-transfected into HEK293 cells. Co-immunoprecipitation was performed as indicated.
F. Y429 of TEAD4 is required for its activation by YAP. Indicated plasmids were co-transfected with a 5×UAS-luciferase reporter and a CMV-β-gal construct. Luciferase activity was measured and normalized to β-galactosidase activity.
G. Y406 in TEAD1 is required for its activation by YAP. Experiments were similar to those in panel E.
H. TEAD1/3/4 shRNAs blocked YAP induced anchorage-independent growth in soft agar. Indicated MCF10A stable cells were plated in soft agar and allowed to grow for 3 weeks, after which colonies were stained with crystal violet and pictured.


A.
Vec
YAP-WT
YAP-S94A
Ki67
DAPI
Relative Ki67
YAP
WT
S94A
Myc
Tubulin
B.
shRNA
Scramble
TEAD1/3/4
Myc-YAP
Vec
5SA
E-Cadherin
gamma-Catenin
Hsp90
C.
H. sapiens
TEAD1 AQHHIY406RLVKD
TEAD2 AQHHIY RLVRD
TEAD3 AQHHVY KLVKD
TEAD4 AQHHIY429RLVKE
D. melanogaster Scalloped TTHHIY RLIKE
D.
YAP
TEAD Binding Domain
WW1
WW2
Activation Domain
S94
Y406
TEA Domain
YAP Binding Domain
TEAD1
E.
Myc-TEAD4
Flag-YAP
Y429H
Y429A
IP: Flag
IP: Myc
Lysate
F.
Control
YAP
Relative Luciferase
Gal4-TEAD4
G.
Gal4-TEAD1
Y406H
H.
Vec
5SA
5SA-S94A
5SA +
Scramble shRNA
TEAD1/3/4 shRNA #1
TEAD1/3/4 shRNA #2

CTGF Is a Direct YAP-TEAD Target Gene Required for Cell Growth

YAP expression affected many cell proliferation related genes. 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. This indicates that there might be different genes in mammalian cells to mediate YAP function. CTGF is highly induced by YAP expression in both 3T3 and MCF10A cells, and its promoter is co-occupied by YAP and TEAD1 as shown by chromatin immunoprecipitation (Fig.3.5A), 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 co-expression (Fig.3.5B). 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.3.5C). These results indicate that YAP activates CTGF promoter through TEAD. Examination of the CTGF promoter region revealed three putative TEAD binding sites (Anbanandam et al. 2006) (Fig.3.5D). Individual or combinatory mutation of the putative TEAD binding sites indicated that the TB2 and TB3 were more important for CTGF promoter activity while TB1 was also involved (Fig.3.5E).

The function of endogenous YAP and TEAD in CTGF expression was examined by YAP or TEAD1/3/4 knockdown 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 (Fig.3.6A) and western blot (Fig.3.5G). We found that knockdown of either YAP or TEAD1/3/4 caused a dramatic reduction of both CTGF mRNA (Fig.3.5F) and protein (Fig.3.5G). 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.3.5H). This data further demonstrates 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-5SA expressing

MCF10A cells decreased the acini growth and reversed the complex shaped and rough surface morphology in 3D culture (Fig.3.5I). 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-5SA overexpressing MCF10A cells. Although CTGF knockdown did not completely block the anchorage-independent growth of YAP-5SA overexpressing MCF10A cells, it significantly decreased the number of colonies formed (Fig.3.5J, 3.6B), and dramatically reduced the colony size (Fig.3.5J). 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.

Figure 3.5 CTGF is a direct target of YAP and TEAD.

A. Both YAP and TEAD1 bind to CTGF promoter. Chromatin immunoprecipitation 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 co-transfected with YAP wild type or S94A mutant as indicated with or without TEAD1 co-transfection. Luciferase activity was measured and normalized to co-transfected β-galactosidase.

C. Dominant negative TEAD1 blocks the YAP stimulation of the CTGF reporter. Indicated plasmids were co-transfected 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 (TB1-3) 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. 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.

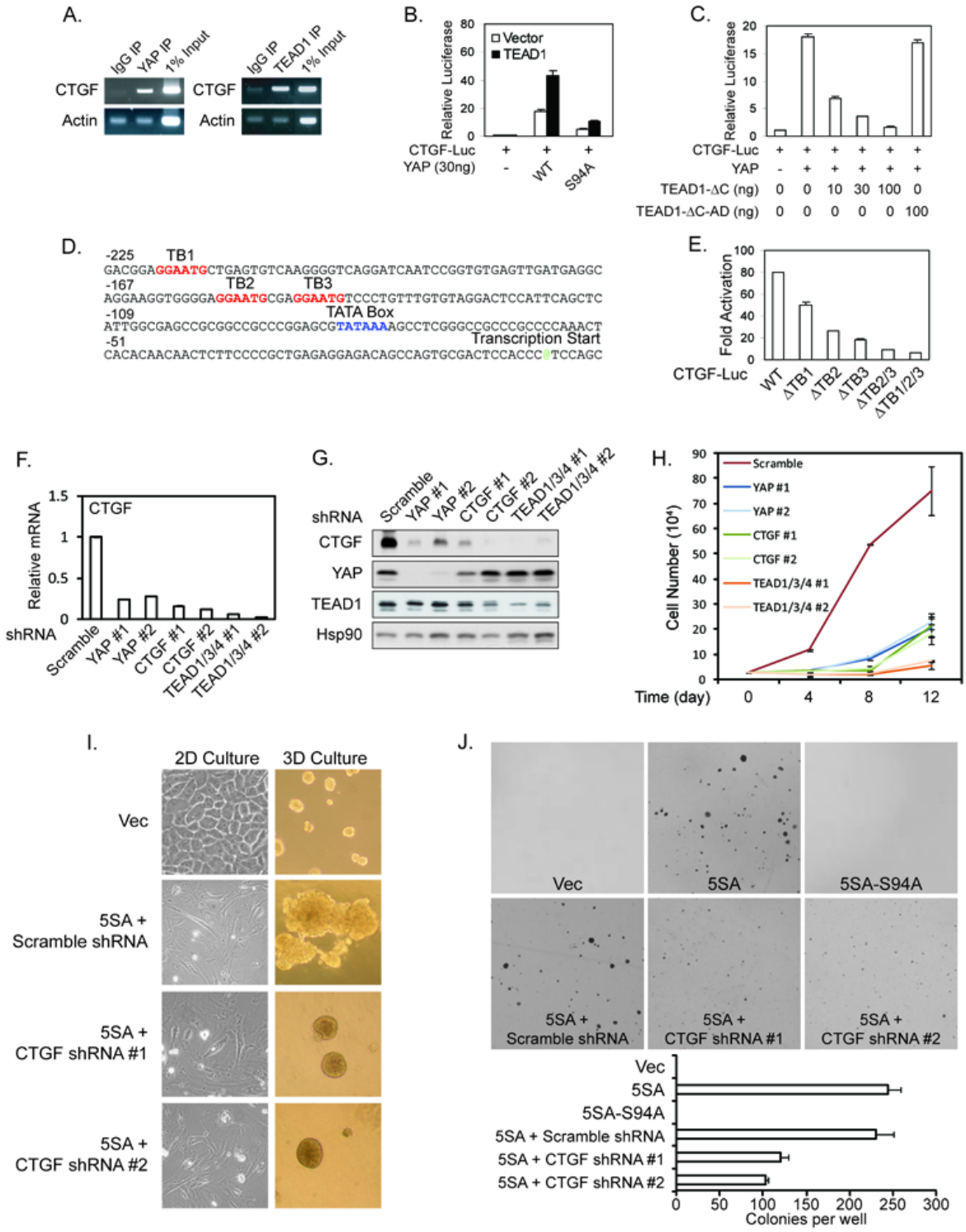
F. YAP and TEAD are required for CTGF expression. ACHN cells were infected with indicated shRNA lentiviruses and CTGF mRNA levels were determined by quantitative RT-PCR.

G. Knockdown of YAP or TEAD1/3/4 decreases CTGF protein levels. Experiments were similar to panel F except Western blotting was performed with 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 is required for YAP induced growth and morphological change in 3D culture. MCF10A cells expressing YAP-5SA were infected with indicated shRNA lentiviruses. The morphology in 2D and 3D culture were documented as in Fig.3.3C.

J. CTGF knockdown attenuates YAP induced anchorage-independent growth in soft agar. Indicated MCF10A stable cells were plated in soft agar and allowed to grow for 3 weeks, 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.


A.
IgG IP
YAP IP
1% Input
CTGF
Actin
IgG IP
TEAD1 IP
1% Input
CTGF
Actin
B.
Relative Luciferase
Vector
TEAD1
CTGF-Luc
YAP (30ng)
WT
S94A
C.
Relative Luciferase
CTGF-Luc
YAP
TEAD1-ΔC (ng)
TEAD1-ΔC-AD (ng)
D.
-225 TB1
GACGGAGGAATGCTGAGTGTCAAGGGGTCAGGATCAATCCGGTGTGAGTTGATGAGGC
-167 TB2 TB3
AGGAAGGTGGGGAGGAATGCGAGGAATGTCCCTGTTTGTGTAGGACTCCATTCAGCTC
-109 TATA Box
ATTGGCGAGCCGCGGCCGCCCGGAGCGTATAAAAGCCTCGGGCCGCCCGCCCCAAACT
-51 Transcription Start
CACACAACAACTCTTCCCCGCTGAGAGGAGACAGCCAGTGCGACTCCACCCTCCAGC
E.
Fold Activation
CTGF-Luc
WT
ΔTB1
ΔTB2
ΔTB3
ΔTB2/3
ΔTB1/2/3
F.
Relative mRNA
CTGF
shRNA
Scramble
YAP #1
YAP #2
CTGF #1
CTGF #2
TEAD1/3/4 #1
TEAD1/3/4 #2
G.
shRNA
CTGF
YAP
TEAD1
Hsp90
H.
Cell Number (10⁴)
Time (day)
Scramble
YAP #1
YAP #2
CTGF #1
CTGF #2
TEAD1/3/4 #1
TEAD1/3/4 #2
I.
2D Culture
3D Culture
Vec
5SA + Scramble shRNA
5SA + CTGF shRNA #1
5SA + CTGF shRNA #2
J.
Vec
5SA
5SA-S94A
5SA + Scramble shRNA
5SA + CTGF shRNA #1
5SA + CTGF shRNA #2
Colonies per well

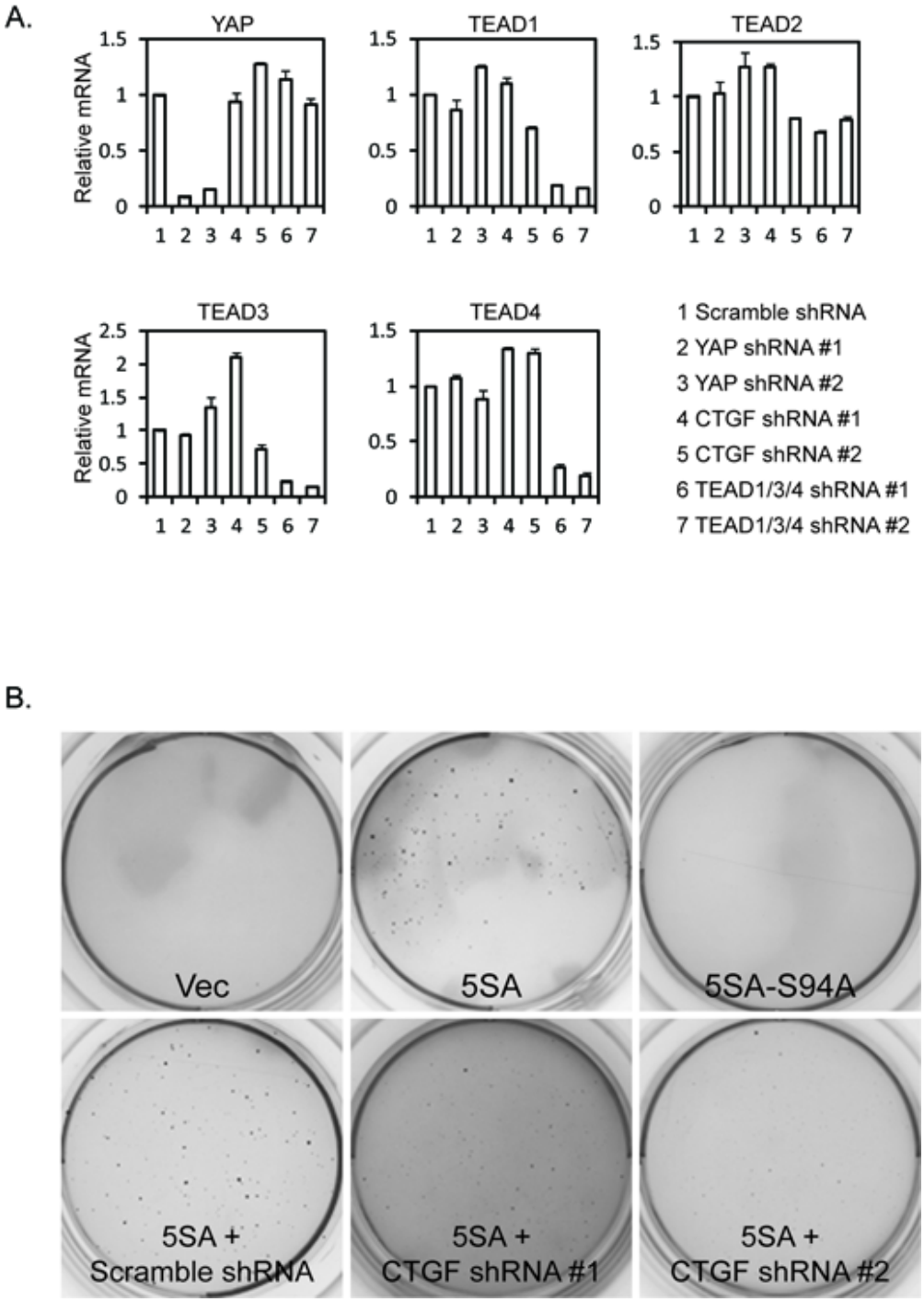


Figure 3.6 Supplemental to CTGF is a direct target of YAP and TEAD.

A. Quantitative RT-PCR was performed to verify the efficiency and specificity of shRNAs against YAP and TEAD1/3/4 in ACHN cells.

B. CTGF knockdown attenuates YAP induced anchorage-independent growth in soft agar. Experiments were the same as the ones in Fig. 3.5J. Pictures were shown in lower magnification to show colonies in whole wells.

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 expressing human YAP-S127A (an active form) or YAP-S94A/S127A in developing eyes. YAP-S127A overexpression significantly increased eye size (Fig.3.8A, a, d) and the number of interommatidial cells (Fig.3.7A, a, d). Mutation of S94A dramatically decreased the activity of YAP-S127A in promoting tissue growth (Fig.3.7A, e; Fig.3.8A, e). Scalloped (Sd) is the only TEAD homolog in *Drosophila*. We found that Yki directly interacted with Sd in an *in vitro* binding assay (Fig.3.8B). 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.3.7A, a-c; Fig.3.8A, 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.3.7B). 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.3.7B, a, b, d). However, neither YAP-S94A/S127A nor Yki-S97A showed similar level of growth-promoting effect (Fig.3.7B, 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.3.7C. a-d). Thus, the level of Sd is critical for Yki to promote tissue growth. Overexpression of Sd caused small eyes (Fig.3.7C, 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.3.7C. f). Furthermore, co-expression of Yki with Sd suppressed the reduced eye phenotype caused by Sd overexpression (Fig.3.7C, 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.

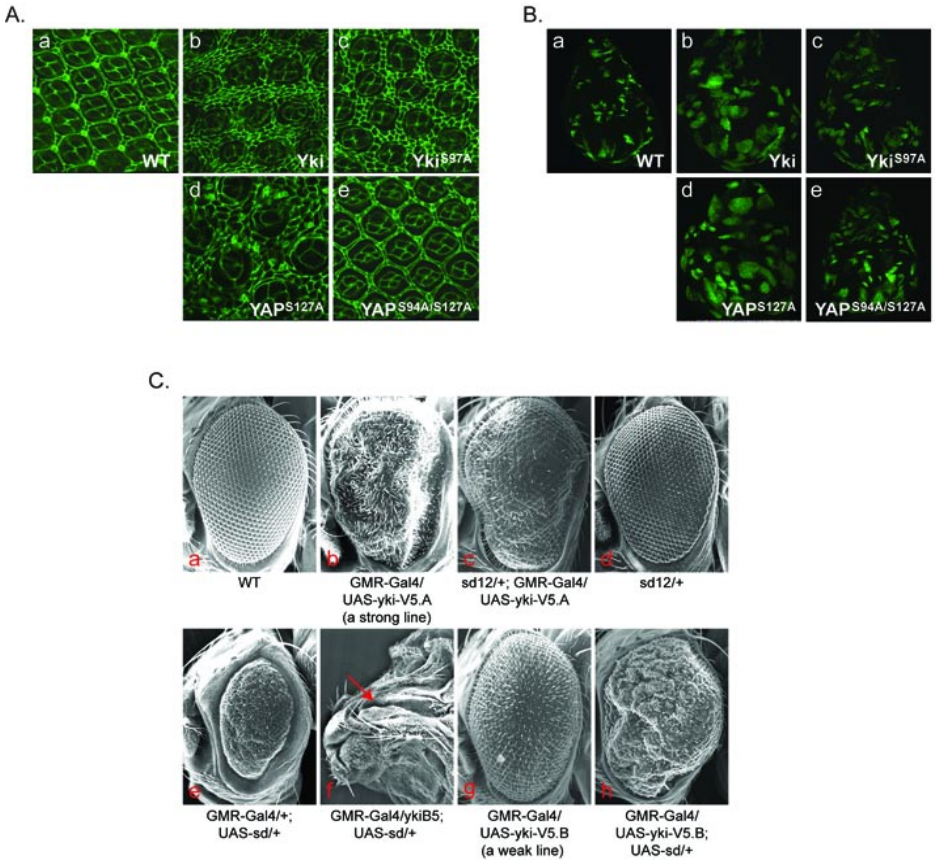


Figure 3.7 *yki* and *scalloped* genetically interact to control tissue growth and organ size.

A. The TEAD/ Sd binding defective YAP and Yki are compromised in inducing extra interommatidial cells. Mid-pupal eye discs were stained with Discs large (Dlg) antibody to outline 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^{S127A}*

(e). *GMR-Gal4/UAS-Flag-YAP$^{S94A/S127A}$*

B. The TEAD/ Sd binding defective YAP and Yki are compromised in inducing clone expansion. Wing imaginal discs containing 72h-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:

(a). *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/+*

(b). *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-yki-V5*

(c). *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-yki^{S97A}-V5*

(d). *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-Flag-YAP^{S127A}*

(e). *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-Flag-YAP$^{S94A/S127A}$*

C. *yki* and *scalloped* genetically interact to control tissue growth and organ size. Genotypes of the fly tissues are indicated. SEM (scanning electron microscopy) images of adult eyes are shown in (a-e and g-h). A late pupal head is shown in (f). The arrow in (f) indicates where a retina is normally expected to grow.

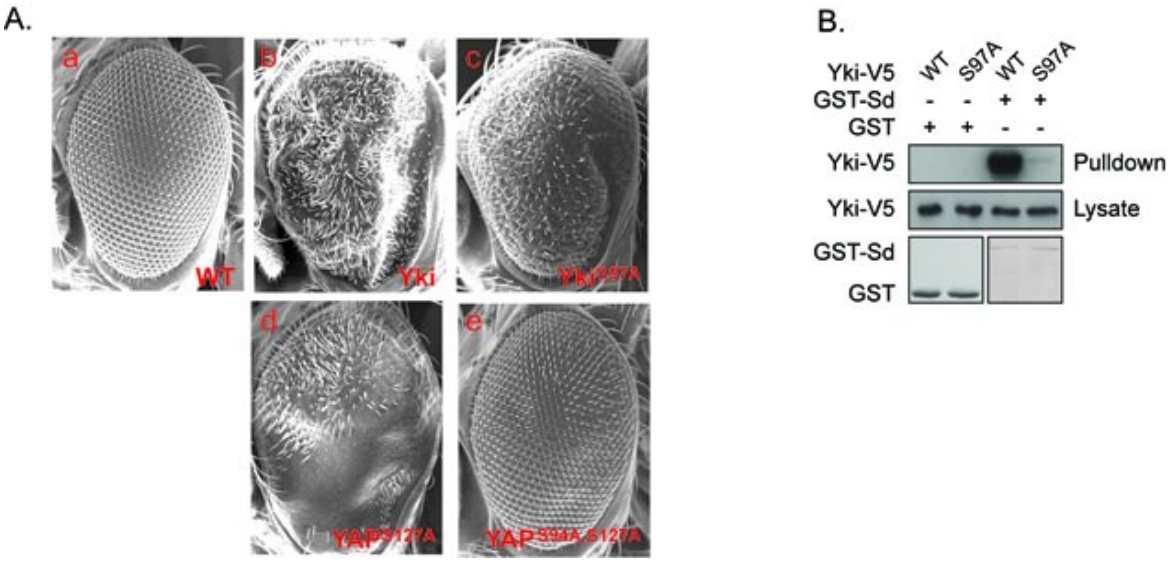


Figure 3.8 Supplemental to *yki* and *scalloped* genetically interact to control tissue growth and organ size.

A. The TEAD/Sd binding defective YAP and Yki are 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^{S127A}*

(e). *GMR-Gal4/UAS-Flag-YAP$^{S94A/S127A}$*

B. Yki but not Yki-S97A interacts with Scalloped. Bacterially produced GST-Sd was used in the *in vitro* Yki pull-down assay. Yki-V5 was produced in transfected S2 cells. Yki was detected by anti-V5 Western blot and GST proteins were detected by Coomassie Blue staining.

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 report, we have 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 of 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 have 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.

Contents of this chapter were published in the following paper: Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Lin, J.D., Wang, C.Y., Chinnaiyan, A.M., Lai, Z.C., and Guan, K.L. 2008. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* **22**(14): 1962-1971.

Bibliography

Anbanandam, A., Albarado, D.C., Nguyen, C.T., Halder, G., Gao, X., and Veeraraghavan, S. 2006. Insights into transcription enhancer factor 1 (TEF-1) activity from the solution structure of the TEA domain. *Proc Natl Acad Sci U S A* **103**(46): 17225-17230.

Basu, S., Totty, N.F., Irwin, M.S., Sudol, M., and Downward, J. 2003. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Mol Cell* **11**(1): 11-23.

Camargo, F.D., Gokhale, S., Johnnidis, J.B., Fu, D., Bell, G.W., Jaenisch, R., and Brummelkamp, T.R. 2007. YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr Biol* **17**(23): 2054-2060.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. 2003. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* **30**(3): 256-268.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S.A., Gayyed, M.F., Anders, R.A., Maitra, A., and Pan, D. 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* **130**(6): 1120-1133.

Dornhofer, N., Spong, S., Bennewith, K., Salim, A., Klaus, S., Kambham, N., Wong, C., Kaper, F., Sutphin, P., Nacamuli, R., Hockel, M., Le, Q., Longaker, M., Yang, G., Koong, A., and Giaccia, A. 2006. Connective tissue growth factor-specific monoclonal antibody therapy inhibits pancreatic tumor growth and metastasis. *Cancer Res* **66**(11): 5816-5827.

Edgar, B.A. 2006. From cell structure to transcription: Hippo forges a new path. *Cell* **124**(2): 267-273.

Fossdal, R., Jonasson, F., Kristjansdottir, G.T., Kong, A., Stefansson, H., Gosh, S., Gulcher, J.R., and Stefansson, K. 2004. A novel TEAD1 mutation is the causative allele in Sveinsson's chorioretinal atrophy (helicoid peripapillary chorioretinal degeneration). *Hum Mol Genet* **13**(9): 975-981.

Goulev, Y., Fauny, J.D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. 2008. SCALLOPED Interacts with YORKIE, the Nuclear Effector of the Hippo Tumor-Suppressor Pathway in Drosophila. *Curr Biol* **18**(6): 435-441.

Hao, Y., Chun, A., Cheung, K., Rashidi, B., and Yang, X. 2007. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *J Biol Chem*.

Hariharan, I.K. and Bilder, D. 2006. Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. *Annu Rev Genet* **40**: 335-361.

Harvey, K. and Tapon, N. 2007. The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. *Nat Rev Cancer* **7**(3): 182-191.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. 2003. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**(4): 457-467.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**(3): 421-434.

Kitagawa, M. 2007. A Sveinsson's chorioretinal atrophy-associated missense mutation in mouse Tead1 affects its interaction with the co-factors YAP and TAZ. *Biochem Biophys Res Commun* **361**(4): 1022-1026.

Komuro, A., Nagai, M., Navin, N.E., and Sudol, M. 2003. WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. *J Biol Chem* **278**(35): 33334-33341.

Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., and Li, Y. 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120**(5): 675-685.

McClatchey, A.I. and Giovannini, M. 2005. Membrane organization and tumorigenesis--the NF2 tumor suppressor, Merlin. *Genes Dev* **19**(19): 2265-2277.

McClatchey, A.I., Saotome, I., Mercer, K., Crowley, D., Gusella, J.F., Bronson, R.T., and Jacks, T. 1998. Mice heterozygous for a mutation at the Nf2 tumor suppressor locus develop a range of highly metastatic tumors. *Genes Dev* **12**(8): 1121-1133.

Moffat, J., Grueneberg, D.A., Yang, X., Kim, S.Y., Kloepfer, A.M., Hinkle, G., Piqani, B., Eisenhaure, T.M., Luo, B., Grenier, J.K., Carpenter, A.E., Foo, S.Y., Stewart, S.A., Stockwell, B.R., Hacohen, N., Hahn, W.C., Lander, E.S., Sabatini, D.M., and Root, D.E. 2006. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* **124**(6): 1283-1298.

Overholtzer, M., Zhang, J., Smolen, G.A., Muir, B., Li, W., Sgroi, D.C., Deng, C.X., Brugge, J.S., and Haber, D.A. 2006. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc Natl Acad Sci U S A* **103**(33): 12405-12410.

Saucedo, L.J. and Edgar, B.A. 2007. Filling out the Hippo pathway. *Nat Rev Mol Cell Biol* **8**(8): 613-621.

Simmonds, A.J., Liu, X., Soanes, K.H., Krause, H.M., Irvine, K.D., and Bell, J.B. 1998. Molecular interactions between Vestigial and Scalloped promote wing formation in Drosophila. *Genes Dev* **12**(24): 3815-3820.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**(43): 15545-15550.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. 2002. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**(4): 467-478.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat Cell Biol* **5**(10): 914-920.

Vassilev, A., Kaneko, K.J., Shu, H., Zhao, Y., and DePamphilis, M.L. 2001. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev* **15**(10): 1229-1241.

Wu, S., Huang, J., Dong, J., and Pan, D. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**(4): 445-456.

Wu, S., Liu, Y., Zheng, Y., Dong, J., and Pan, D. 2008. The TEAD/TEF Family Protein Scalloped Mediates Transcriptional Output of the Hippo Growth-Regulatory Pathway. *Dev Cell*.

Xie, D., Nakachi, K., Wang, H., Elashoff, R., and Koeffler, H.P. 2001. Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. *Cancer Res* **61**(24): 8917-8923.

Yagi, R., Chen, L.F., Shigesada, K., Murakami, Y., and Ito, Y. 1999. A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. *Embo J* **18**(9): 2551-2562.

Yu, J., Rhodes, D.R., Tomlins, S.A., Cao, X., Chen, G., Mehra, R., Wang, X., Ghosh, D., Shah, R.B., Varambally, S., Pienta, K.J., and Chinnaiyan, A.M. 2007. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. *Cancer Res* **67**(22): 10657-10663.

Zender, L., Spector, M.S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S.T., Luk, J.M., Wigler, M., Hannon, G.J., Mu, D., Lucito, R., Powers, S., and Lowe, S.W. 2006. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* **125**(7): 1253-1267.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. 2008. The TEAD/TEF Family of Transcription Factor Scalloped Mediates Hippo Signaling in Organ Size Control. *Dev Cell*.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.C., and Guan, K.L. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* **21(**21): 2747-2761.

CHAPTER 4

WW DOMAINS ARE REQUIRED FOR THE GROWTH STIMULATION AND ONCOGENIC TRANSFORMATION ACTIVITY OF YAP

Abstract

The YAP transcription co-activator 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 homolog, 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.

Introduction

Yes-associated protein (YAP) is a transcription co-activator and a candidate human oncogene regulated by the Hippo pathway, a novel tumor suppressor pathway first

characterized by *Drosophila* genetic studies (Tao et al. 1999; Harvey et al. 2003; Udan et al. 2003; Wu et al. 2003; Lai et al. 2005; Edgar 2006; Hariharan 2006; Hariharan and Bilder 2006; Harvey and Tapon 2007; Saucedo and Edgar 2007). The Hippo pathway limits organ size in *Drosophila* by inhibiting Yki, the YAP homolog (Huang et al. 2005). Biochemical studies demonstrated that Yki is directly phosphorylated and inhibited by the Wts protein kinase, which is phosphorylated and activated by the Hippo (Hpo) protein kinase (Dong et al. 2007; Oh and Irvine 2008). Components of the Hippo pathway are highly conserved in mammals. Recent studies from our group and others have demonstrated that YAP is phosphorylated and inhibited by the Lats tumor suppressor kinase, which is the mammalian homolog of Wts (Hao et al. 2007; Zhao et al. 2007; Zhang et al. 2008a). Lats phosphorylates YAP on serine residue 127 in the HXRXXS motif which results in 14-3-3 binding and cytoplasmic retention of YAP, therefore, leading to YAP inhibition (Zhao et al. 2007). This mechanism of YAP regulation is implicated in cell contact inhibition and tissue growth control (Zhao et al. 2007; Zeng and Hong 2008).

YAP is a potent growth promoter. Overexpression of YAP increases organ size in *Drosophila* and saturation cell density in NIH-3T3 cell culture (Zhao et al. 2007). 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 (Modena et al. 2006; Overholtzer et al. 2006; Zender et al. 2006; Yokoyama et al. 2008). Besides the genomic amplification, YAP expression and nuclear localization were also shown to be elevated in multiple types of human cancers (Zender et al. 2006; Dong et al. 2007; Zhao et al. 2007; Steinhardt et al. 2008). 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 (Overholtzer et al. 2006); YAP cooperates with *myc* oncogene to stimulate tumor growth in nude mice (Zender et al. 2006); and more interestingly, transgenic mice with liver-specific YAP overexpression show a dramatic increase in liver size and eventually develop tumors (Camargo et al. 2007; Dong et al. 2007). 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 co-activator, which itself has no DNA binding activity. Recent studies from *Drosophila* and mammalian cells have demonstrated that TEAD plays a critical role in mediating YAP-dependent gene induction and growth control (Vassilev et al. 2001; Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008b; Zhao et al. 2008). YAP and TEAD bind to a common set of promoters in MCF10A cells (Zhao et al. 2008). 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 (Zhao et al. 2008). The *Drosophila* TEAD homolog, Scalloped (Sd), also interacts with Yki and is required for Yki to stimulate tissue growth (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008b). 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 (Paumard-Rigal et al. 1998; Simmonds et al. 1998; Huang et al. 2005), and overexpression of the Sd-binding-defective Yki-S97A elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (Zhao et al. 2008). Consistently, the TEAD-binding-defective YAP-S94A mutant can still induce expression of a fraction of YAP regulated genes (Zhao et al. 2008). These observations indicate that besides TEAD, additional transcription factors may be used by YAP/Yki to stimulate cell and tissue growth.

YAP has an N-terminal TEAD binding domain (TBD) and a C-terminal transactivation domain, with one or two WW domains (two splicing variants, YAP1 and YAP2, respectively) in between (Sudol et al. 1995). The WW domain is known to be a protein-protein interaction module with two signature tryptophan (W) residues spaced 20-22 amino acids apart (Sudol and Hunter 2000). 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 (Komuro et al. 2003), RUNX2 (Yagi et al. 1999), and p73 (Strano et al. 2001; Basu et al. 2003) 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 (Hao et al. 2007; Oka et al. 2008).

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 while the 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

Mthods described in Chapter 2 and Chapter 3 is not repeated.

Antibodies, Plasmids, and Materials

FITC mouse anti-E-Cadherin was obtained from BD Biosciences (San Jose, CA). Anti-alpha-tubulin was obtained from Sigma (St. Louis, MO). Anti-Myc antibody was obtained from Covance (Philadelphia, PA). Rhodamine phalloidin was obtained from Invitrogen (Carlsbad, CA). Horseradish peroxidase conjugated secondary antibodies were obtained from Amersham (Buckinghamshire, UK).

The pCMV-Flag-YAP2 and the pM-ErbB4-CTFΔK constructs were kindly provided by Dr. Marius Sudol, and YAP2 was subcloned into the pQCXIH-Myc retrovirus vector. The YAP-5SA, 4SA, S127A, S94A, W1, W2, and W1W2 mutants were generated by site-directed mutagenesis. To mutate the WW domain, the second critical tryptophan and its plus 3 position proline were mutated to alanine. pcDNA3-HA-Lats2 was a gift from Dr. Tian Xu. Lats2-YA mutant was generated by mutate the critical tyrosine residue in the PPXY motif to alanine. Mob was cloned from human brain cDNA library into the pcDNA3-HA vector. The RUNX2 and 6×OSE2-luc reporter were from Dr. Hongjiao Ouyang. The 5×UAS-luciferase reporter and the Gal4-TEAD4 construct were described before.

Immunohistochemical Staining

The lung cancer and liver cancer tissue microarrays (TMA) were purchased from US Biomax (Ijamsville, MD). The TMAs were deparaffinized through graded ethanol solutions. After an antigen retrieval procedure of 30 min using target retrieval solution (DAKO, Carpintera, CA), the sections were stained using the avidin-biotin complex system (Vector Laboratory, Burlingame, CA). Step-one reagent was rabbit anti-YAP antibody. Biotinylated goat anti-rabbit antibody and the horseradish peroxidase-ABC system (Vector) were used as second and third-step reagents. 3, 3'-diaminobenzidine (DAB) was used as substrate. Cell nuclei were counterstained with Hematoxylin.

Colony 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 wild-type or mutants using Fugene6 (Roche) according to the manufacturer’s instructions. After 2 days, cells were re-plated onto 10 cm dish and were maintained in DMEM supplemented with 5% fetal bovine serum for 2-3 weeks till foci

were evident. Cells were fixed with 10% acetic acid and 10% methanol, and then colonies were stained with 1% crystal violet and counted.

Drosophila Genetics

For *in vivo* functional analysis of YAP/Yki, full-length cDNAs of *YAP* or *yki* were cloned into a transformation vector *pUAST* (Brand and Perrimon 1993). Multiple transgenic fly lines were generated for each of the following DNA constructs: *pUAS-Flag-YAP*$^{S127A/W1W2}$ (15 lines), and *pUAS-yki*^{W1W2}*-V5* (31 lines). *pUAS-Flag-YAP*^{S127A} and *pUAS-yki-V5* were previously reported (Zhao et al. 2007). *C5-Gal4* and *GMR-Gal4* drive wing-specific 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 one hour and then left at 20°C for another three days. Late third instar larvae were dissected and wing imaginal discs were fixed in 8% paraformaldehyde-lysine-phosphate (PLP) buffer for 45 minutes at 4°C. GFP signal was observed by confocal microscopy. Immunofluorescent staining of mid-pupal eye discs was done with mouse anti-Discs large (Dlg) (DSHB, 1:300) as primary antibody and Alexa Fluor 488 (Molecular Probes, 1:300) 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 (Hao et al. 2007; Oka et al. 2008). To test this possibility, we examined the effect of Lats on YAP WW domain mutant in reporter assay. Our data shows that with Mob co-transfection, Lats could potently inhibit both wild type and WW domain mutant YAP (Fig.4.1A), indicating that

the WW domains of YAP are not required for its inhibition by Lats. Similar results were obtained without Mob co-transfection, 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.4.1A). 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 RUNX2, have been reported to be activated by YAP (Yagi et al. 1999; Komuro et al. 2003). 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 (Zhao et al. 2008). As expected, S94A mutation of YAP completely abolished its ability to activate TEAD4 (Fig.4.1B). Interestingly, YAP S94A mutant 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 (Yagi et al. 1999; Komuro et al. 2003). We found that mutation of the first (W1) or both (W1W2) WW domains in YAP abolished its ability to activate ErbB4 or RUNX2 while mutation of the second WW domain (W2) only modestly decreased this activity (Fig.4.1B). However, mutation of the WW domains does not attenuate the activity of YAP on TEAD4. These data indicate that YAP utilizes 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 (Zhao et al. 2008). Here we compared gene expression profiles of MCF10A cells overexpressing YAP wild-type or WW domain mutant. Interestingly, a subset of YAP inducible genes requires the intact WW domains in YAP (Table 4.1). 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 TEAD binding but not WW domains. Moreover, induction of ITGB2 and PIK3C2B require both the TBD and WW domains (Fig.4.1C). 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.

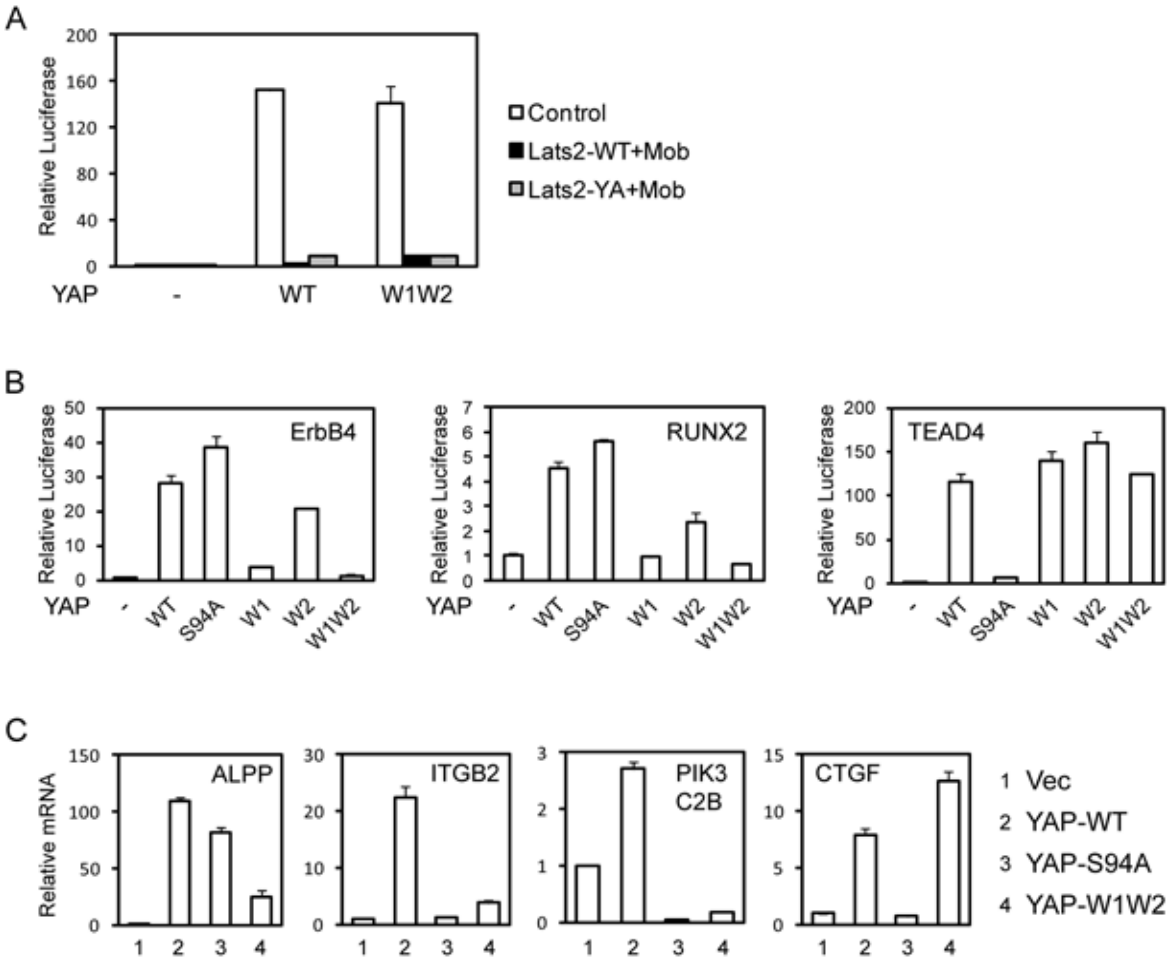


Figure 4.1 WW domains of YAP mediate gene induction but are not required for YAP inhibition by Lats.

A. WW domains of YAP are not required for the inhibition by Lats2. Indicated plasmids were co-transfected 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 wild-type or mutants were co-transfected with the indicated transcription factors into 293T cells. ErbB4 intracellular domain and TEAD4 were Gal4-fused and were co-transfected with a 5×UAS-luciferase reporter. RUNX2 was co-transfected with the 6×OSE2-luciferase reporter. Luciferase activity was measured and normalized to activity of co-transfected β-galactosidase.

C. Both the TBD and WW domains are involved in YAP induced gene expression. MCF10A cells stably expressing YAP wild-type or mutants were generated by retroviral infection. The expression of indicated genes was determined by quantitative RT-PCR and compared to vector control cells.

Gene Symbol	Gene Title	WT/Control	S94A/Control	W1W2/Control	WT/W1W2
FLJ37035	FLJ37035 protein	4.00	1.48	0.14	28.52
TOX2	TOX high mobility group box family member 2	16.62	0.93	0.69	24.04
SPIN3	spindlin family, member 3	8.43	6.34	0.37	23.10
RNF165	ring finger protein 165	23.51	6.48	1.17	20.12
ALPP	alkaline phosphatase, placental (Regan isozyme)	57.41	0.54	3.28	17.50
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	18.19	12.84	1.09	16.63
PHLDB2	Pleckstrin homology-like domain, family B, member 2	8.43	8.63	0.57	14.66
PRSS7	protease, serine, 7 (enterokinase)	22.19	22.44	1.72	12.91
RAB5A	RAB5A, member RAS oncogene family	5.99	3.66	0.60	10.06
KCNT2	potassium channel, subfamily T, member 2	12.16	0.19	1.23	9.92
NT5DC4	5'-nucleotidase domain containing 4	10.01	3.49	1.13	8.86
GPR98	G protein-coupled receptor 98	4.54	2.87	0.54	8.40
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	5.67	0.13	0.78	7.25
ITGB2	integrin, beta 2	31.45	3.69	4.34	7.25
KIAA2018	KIAA2018	8.22	6.63	1.44	5.69
TRIO	triple functional domain (PTPRF interacting)	12.53	6.11	2.24	5.60
LOXL2	lysyl oxidase-like 2	7.43	3.63	1.71	4.34
PRSS23	protease, serine, 23	5.09	0.62	1.18	4.32
MIER3	mesoderm induction early response 1, family member 3	14.93	4.41	3.57	4.18
OR1E1 /2	olfactory receptor, family 1, subfamily E, member 1 /// member 2	5.26	4.73	1.30	4.06
TCEAL2	transcription elongation factor A (SII)-like 2	4.74	1.06	1.18	4.02
CLIC3	chloride intracellular channel 3	8.64	0.83	2.25	3.85
C10orf56	chromosome 10 open reading frame 56	7.27	2.31	1.94	3.74
TNS1	tensin 1	6.61	0.74	1.81	3.64
IGHV1-69	immunoglobulin heavy variable 1-69	4.79	5.70	1.37	3.50
SCARA3	scavenger receptor class A, member 3	51.92	7.36	14.91	3.48
EZR	ezrin	5.56	2.28	1.68	3.31
KRT4	keratin 4	4.00	0.21	1.25	3.19
HEG1	HEG homolog 1 (zebrafish)	6.91	0.53	2.17	3.18
L1CAM	L1 cell adhesion molecule	4.02	1.66	1.30	3.09
FGF9	fibroblast growth factor 9 (glia-activating factor)	5.57	1.24	1.83	3.04
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	4.66	1.15	1.57	2.96
SLC7A1	solute carrier family 7 , member 1	4.28	1.22	1.72	2.49
ANKRD28	ankyrin repeat domain 28	4.17	0.88	1.73	2.41
ASPM	asp (abnormal spindle) homolog, microcephaly associated	4.12	2.77	1.77	2.33
DNM3	dynamin 3	4.05	0.74	1.87	2.16

Table 4.1 Gene expression profiling of WW domain dependent YAP target genes. Genes that are induced or repressed by wild-type YAP overexpression above a two-fold cut-off were defined as YAP target genes. From this list, WW domain dependent genes were sorted-out if they meet one of the following criteria: first, YAP-WT expression induced the gene more than three-fold compared with YAP-W1W2 expression; second, YAP-W1W2 induced the gene less than two-fold. The induction fold of these genes by YAP-S94A expression was also listed. The gene list was ranked by the fold difference comparing YAP-WT and YAP-W1W2.

The WW Domain Is Required for YAP Induced Proliferation but Not EMT

YAP expression stimulates cell growth in both NIH-3T3 fibroblast and MCF10A (Overholtzer et al, 2006; Zhao et al, 2008), a human mammary epithelial cell line. We tested the function of YAP WW domains in stimulating cell growth. Stable expression of wild type YAP significantly increased NIH-3T3 cell growth compared to the vector control cells (Fig.4.2A). 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.4.2B). In contrast, mutation of WW domains significantly attenuated this activity of YAP-5SA.

Previously, it had been reported that YAP expression promotes epithelial-mesenchymal transition (EMT) in MCF10A cells (Zhao et al. 2008). 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 morphological changes similar to those induced by YAP-5SA (Fig.4.2C). In contrast, mutation of S94 or deletion of the C-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.4.2D). These alterations were induced by wild type YAP as well as YAP-W1W2 but not YAP-S94A mutant (Fig.4.2D). 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.

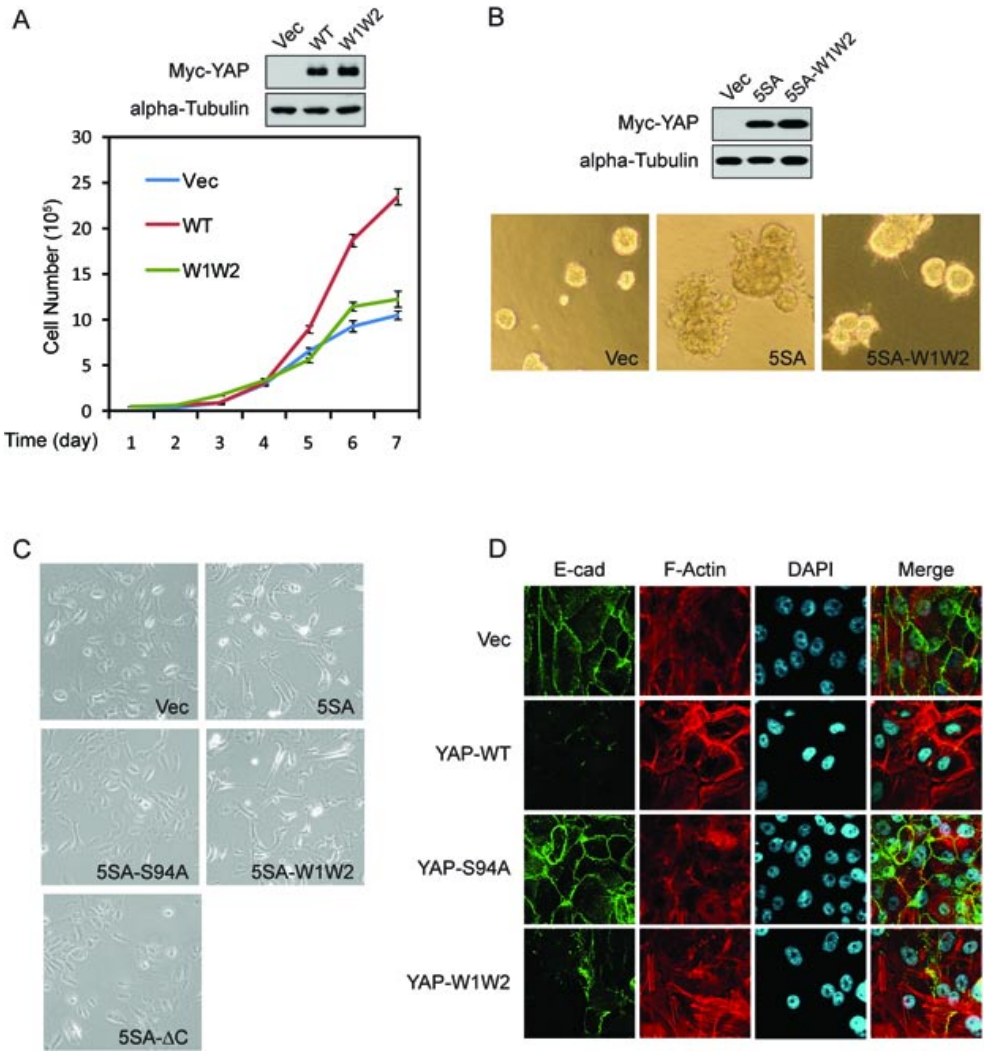


Figure 4.2 The WW domain is required for YAP induced overgrowth but not EMT.

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. The expression of YAP wild-type or W1W2 mutant was shown by western blot (upper panel).

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 days before pictures were taken. The ectopic expression of YAP was shown by western blot (upper panel).

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 DAPI (blue).

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 (Hanahan and Weinberg 2000). We tested the ability of YAP wild-type 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 wild type or active forms of YAP confers NIH-3T3 cells proliferation potential in low serum medium (Fig.4.3A, 4.3B). This is consistent with the oncogenic function of YAP. In contrast, the TBD or WW domain defective mutants completely lost the ability to promote serum independent growth. In fact, under low serum conditions, the YAP-S94A or W1W2 expressing cells displayed a significant decrease in cell numbers, likely due to apoptosis, while the vector control cells remain viable (Fig.4.3A, 4.3B). However, under normal culture conditions (10% serum), neither YAP-S94A nor YAP-W1W2 expression induced cell death. These results demonstrate that both the TBD and WW domains are essential for YAP to promote self-sufficiency of growth signals in NIH-3T3 cells.

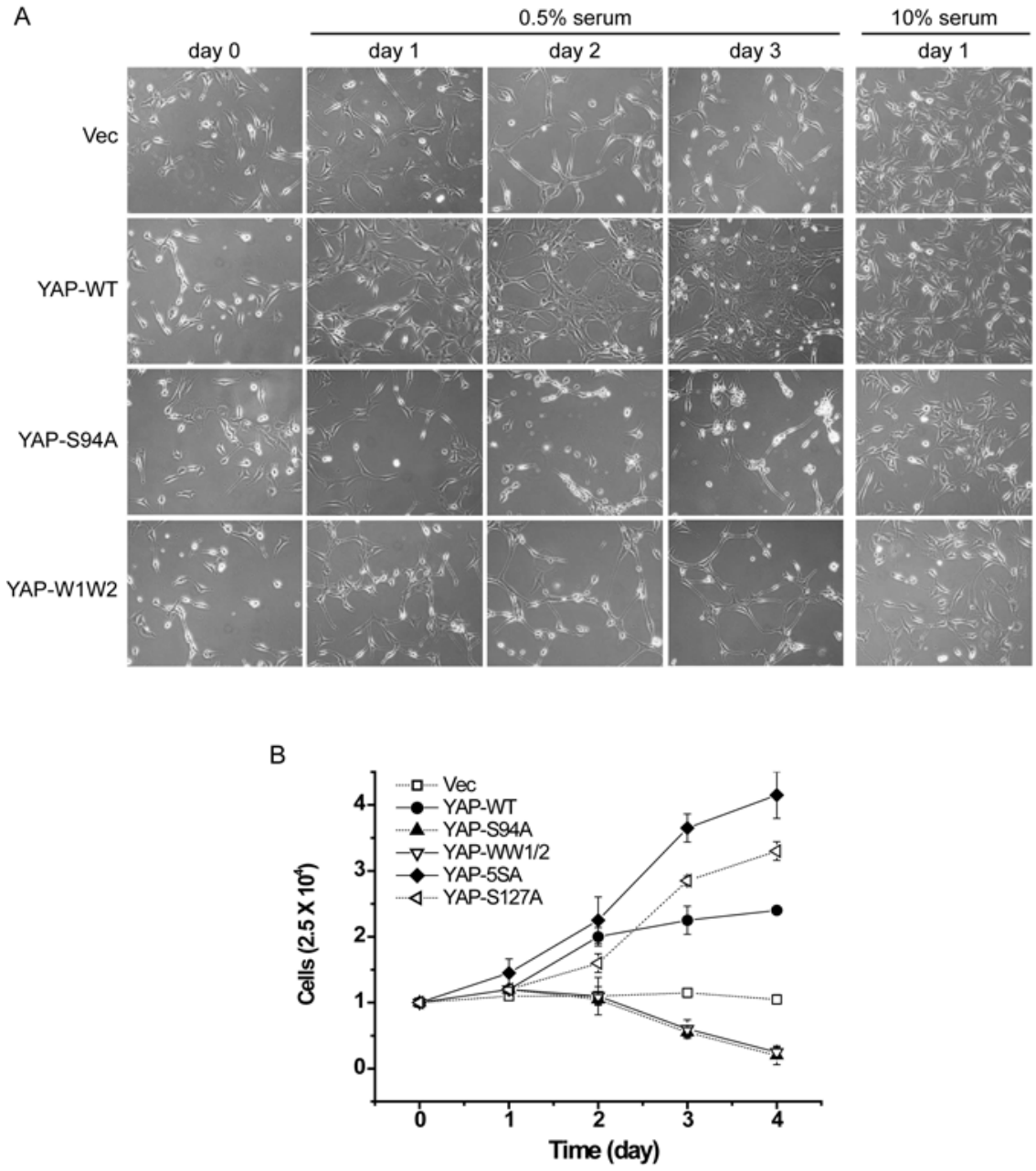


Figure 4.3 Both the TEAD binding and WW domains are required for YAP induced serum-independent growth of fibroblasts.

Stable pools of NIH-3T3 fibroblasts expressing vector control (Vec) and the indicated YAP mutant proteins (wild-type, 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 (2.5×10^4 cells) and then their morphology (A) as well as growth rate (B) were monitored.

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 (Modena et al. 2006; Overholtzer et al. 2006; Zender et al. 2006; Yokoyama et al. 2008). Elevated YAP expression and nuclear localization is also observed in human cancers (Fig.4.4A). 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 wild-type YAP does not induce a transforming morphology (Fig.4.5A). We have previously showed that Lats phosphorylates YAP to inhibit its transactivation and growth promotion activity (Zhao et al. 2007). 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 (Zhao et al. 2007). Interestingly, YAP-5SA is not only more potent in stimulating cell proliferation but also causes transformation properties in NIH-3T3 cells (Fig.4.3B, 4.5A), such as growing on top of each other, indicating the loss of contact inhibition.

We further performed colony formation assays, which is well-established to examine oncogenic potential. As expected, YAP-5SA could potently induce colony formation while YAP wild-type could not (Fig.4.5B, 4.5C), which indicates 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 61 (4SA/S61), 109 (4SA/S109) and 164 (4SA/S164) did not abolish the transforming activity of YAP-5SA. 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 inhibition, YAP-S127A single site mutant is not able to transform NIH-3T3 cells (Fig.4.5C).

S127 of YAP is directly phosphorylated by Lats (Zhao et al. 2007). We performed *in vitro* kinase assay to test if S381 is also a direct Lats target site. Lats could potently phosphorylate wild-type YAP, but has little activity towards YAP-5SA (Fig.4.5D). All YAP-4SA 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 phospho-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 (Fig.4.4B). The impaired YAP phosphorylation in ACHN is likely due to mutation of Sav, a key component of the Hippo pathway (Tapon et al. 2002). 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.

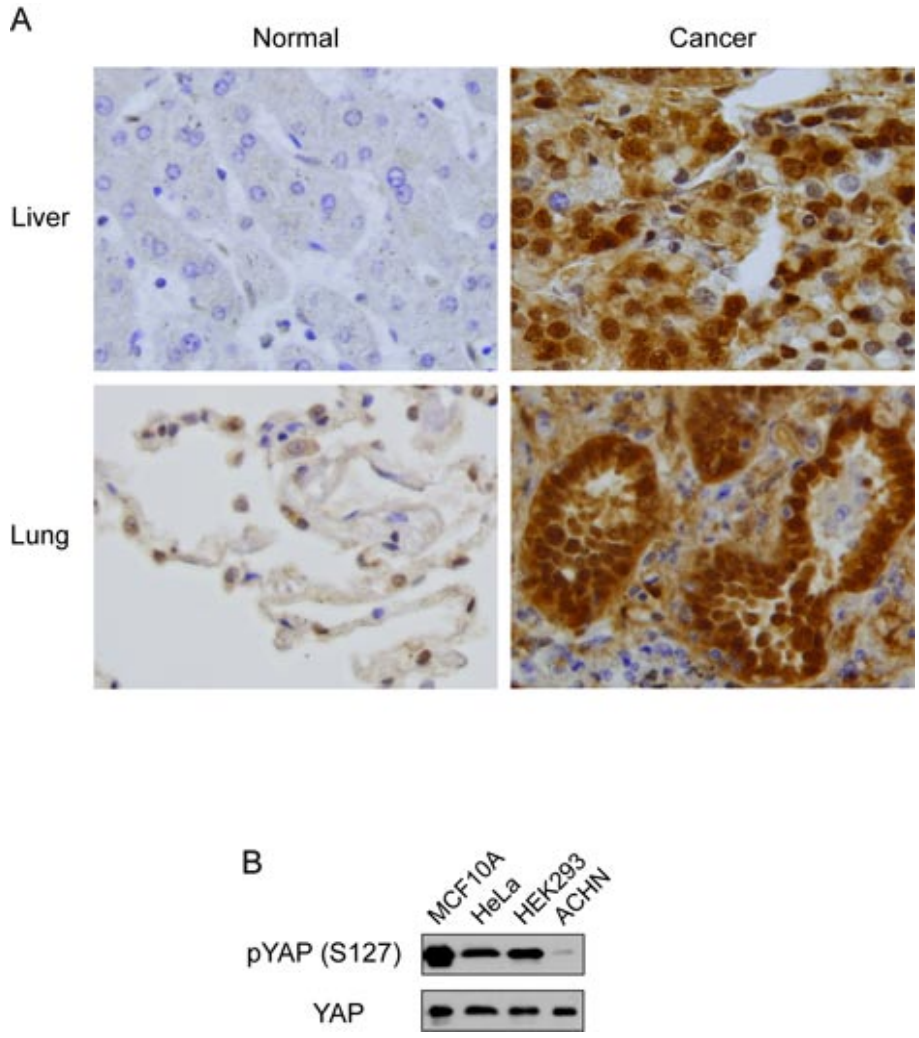


Figure 4.4 Dysregulation of YAP in cancer.

A. Elevated YAP protein level and nuclear localization in human cancers. Tissue microarrays of liver and lung cancer were stained with anti-YAP antibody (brown). Cell nuclei were counterstained with Hematoxylin (blue).

B. YAP phosphorylation is impaired in ACHN cancer cell line. Indicated cells were cultured to confluent before harvest. YAP phosphorylation and protein levels were shown by western blot.

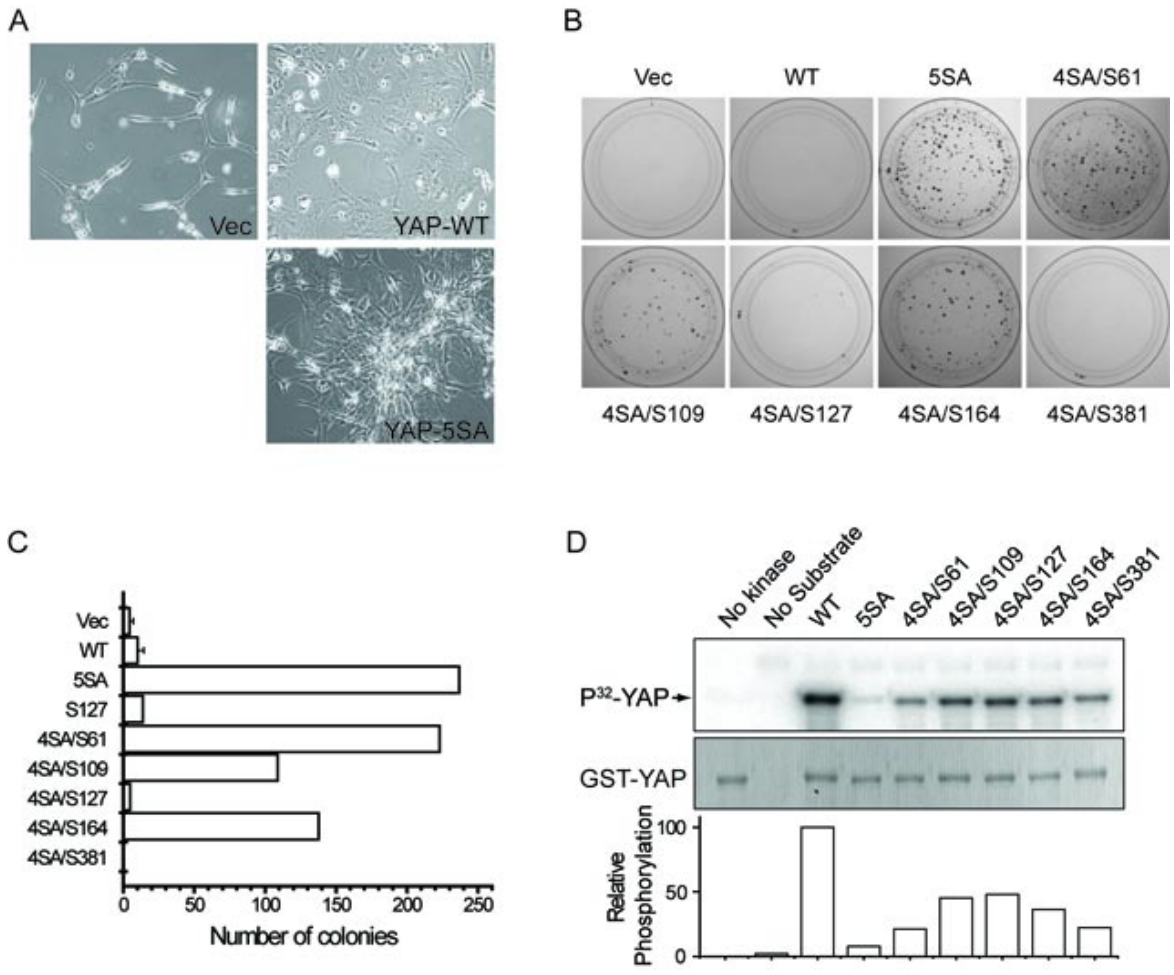


Figure 4.5 Phosphorylation of Serine 127 or 381 is sufficient to inhibit transformation potential of YAP.

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 days 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.

C. Quantification of the colony number shown in panel 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*. Wild-type YAP and various phosphorylation mutants were purified from bacteria as GST-fusion proteins and were subjected to kinase assays in the presence of ^{32}P-ATP with immunoprecipitated Lats from HEK293 cells. Phosphorylation of YAP was detected by ^{32}P incorporation (top panel) and GST-YAP input was shown by Coomassie Blue staining (middle panel). The relative ^{32}P-incorporation was quantified (bottom panel).

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-5SA 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, wild type, S94A, and W1W2 mutant YAP could not transform NIH-3T3 cells (Fig.4.6A, 4.6B). 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.

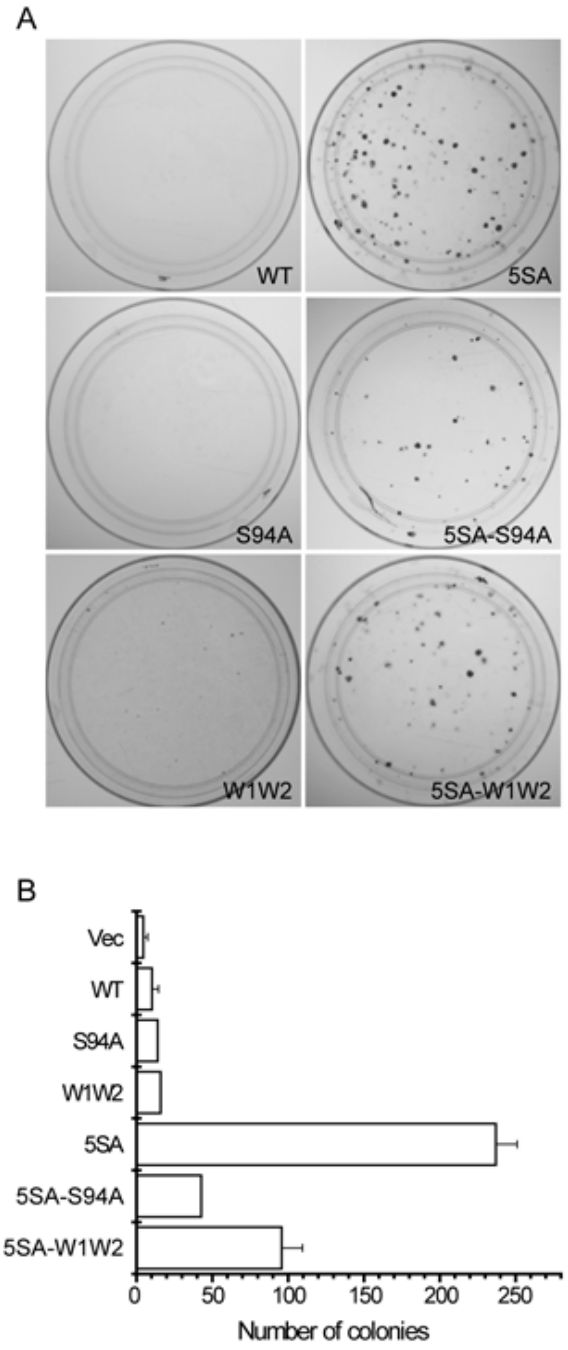


Figure 4.6 Both the TBD and WW domains are important for the oncogenic activity of YAP.
Colony formation assay was performed with indicated plasmids. Colonies were stained with crystal violet and then pictured (A) and counted (B).

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.4.7A, panels a and f; 4.8A). Morphology of four percent 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 to the control flies (Fig.4.7A, panels g and h; 4.8A). 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.4.7A, panel f). This phenotype was not observed in YAP-S94A or YAP-W1W2 flies (Fig.4.7A, panels 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.4.7A, panel 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.4.7A, panels j and k; S2A). In case of fly Yki, its overexpression significantly increased the wing size by 27% (Fig.4.7A, panel b; 4.8A) and about 80% of the wings were too malformed to be measured correctly (Fig.4.7A, panel 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 wild-type controls (Fig.4.7A, panel c and d; 4.8A). 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 to wild-type controls, both Yki and YAP-S127A strongly stimulated clone expansion so that individual clones as well as the entire wing discs were larger (Fig.4.7B, panels 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 wild-type controls (Fig.4.7B, panels 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.4.7C, panels a-e). As expected, they were also less potent than Yki and YAP-S127A, respectively, in increasing the number of interommatidial cells (Fig.4.8B, panels a-e). These results further support our hypothesis that WW domains are important for the growth-promoting activity of Yki and YAP.

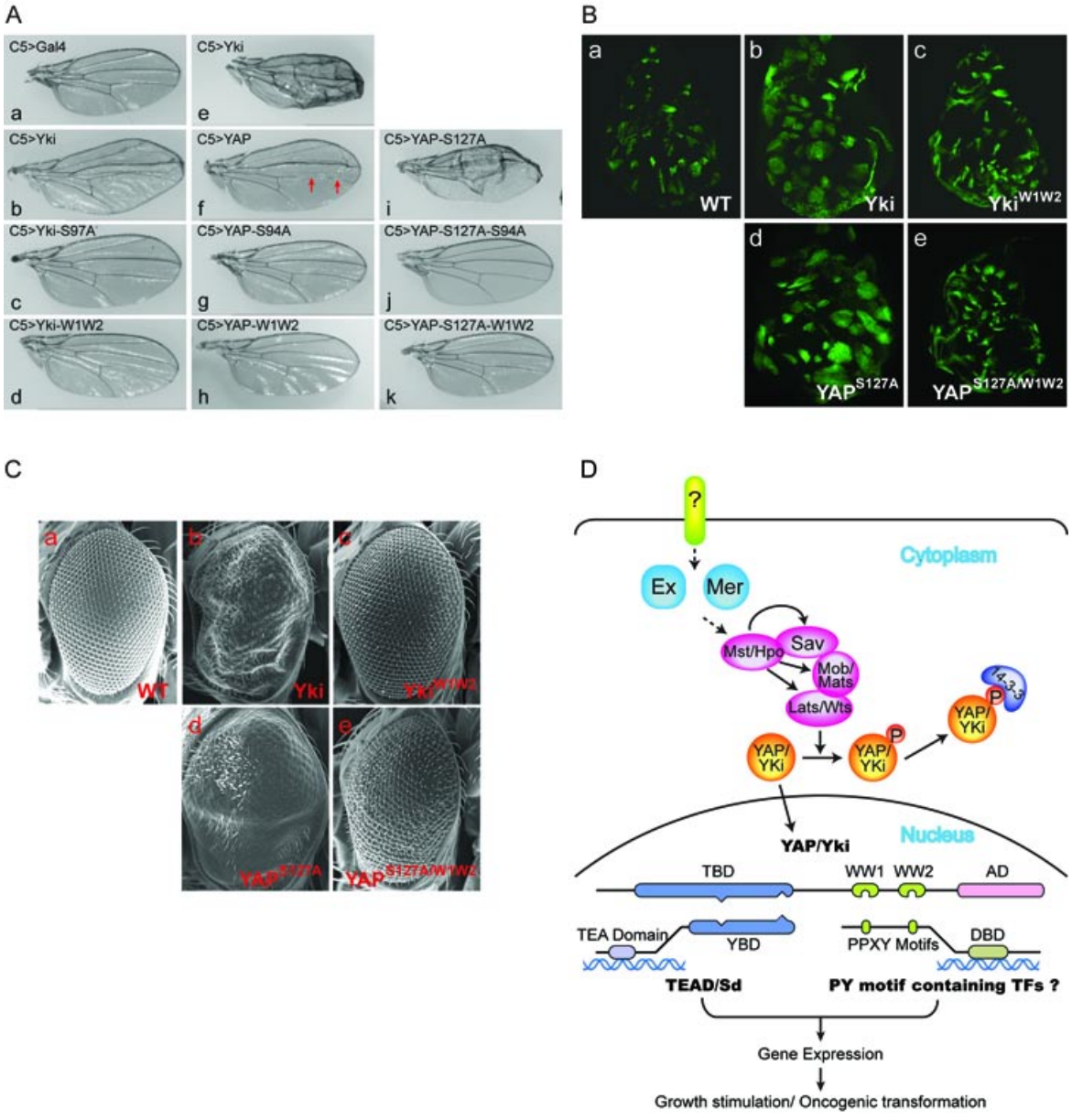
Figure 4.7 The WW domain plays a critical role in YAP/Yki induced tissue growth.

A. The TBD and WW domain mutants of YAP/Yki are compromised in promoting wing tissue growth. Overexpresssion of various *yki* and *yap* transgenes were driven by C5-Gal4. Genotypes of the fly tissues are indicated. In panel f, arrows indicate 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 72h-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-GFP^{S65T}/+* (panel *a*), *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-yki-V5* (panel *b*), *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-yki^{W1W2}-V5* (panel *c*), *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-Flag-YAP^{S127A}* (panel *d*), and *hsFLP/+; act>y+>Gal4, UAS-GFP^{S65T}/UAS-Flag-YAP$^{S127A/W1W2}$* (panel *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 wild-type (Canton S) (panel *a*), *GMR-Gal4/UAS-yki-V5* (panel *b*), *GMR-Gal4/UAS-yki^{W1W2}-V5* (panel *c*), *GMR-Gal4/UAS-Flag-YAP^{S127A}* (panel *d*), and *GMR-Gal4/UAS-Flag-YAP$^{S127A/W1W2}$* (panel *e*).

D. The proposed model of YAP/Yki transcription factor interaction under negative regulation by the Hippo pathway. YAP/Yki interact 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, therefore, stimulates growth and promotes oncogenic transformation. TBD, YBD, DBD, and AD stands for TEAD binding domain, YAP binding domain, DNA binding domain, and activation domain, respectively. For the Hippo pathway components, their name in both mammals and *Drosophila* are given if different. Dashed arrows indicate unknown biochemical mechanisms.


A
C5>Gal4
a
C5>Yki
e
C5>Yki
b
C5>YAP
f
C5>YAP-S127A
i
C5>Yki-S97A
c
C5>YAP-S94A
g
C5>YAP-S127A-S94A
j
C5>Yki-W1W2
d
C5>YAP-W1W2
h
C5>YAP-S127A-W1W2
k
B
a
WT
b
Yki
c
Yki W1W2
d
YAP S127A
e
YAP S127A/W1W2
C
a
WT
b
Yki
c
Yki W1W2
d
YAP S127A
e
YAP S127A/W1W2
D
?
Cytoplasm
Ex
Mer
Mst/Hpo
Sav
Mob/
Mats
Lats/Wts
YAP/
YKi
P
14-3-3
YAP/Yki
Nucleus
TBD
WW1
WW2
AD
TEA Domain
YBD
PPXY Motifs
DBD
TEAD/Sd
PY motif containing TFs ?
Gene Expression
Growth stimulation/ Oncogenic transformation

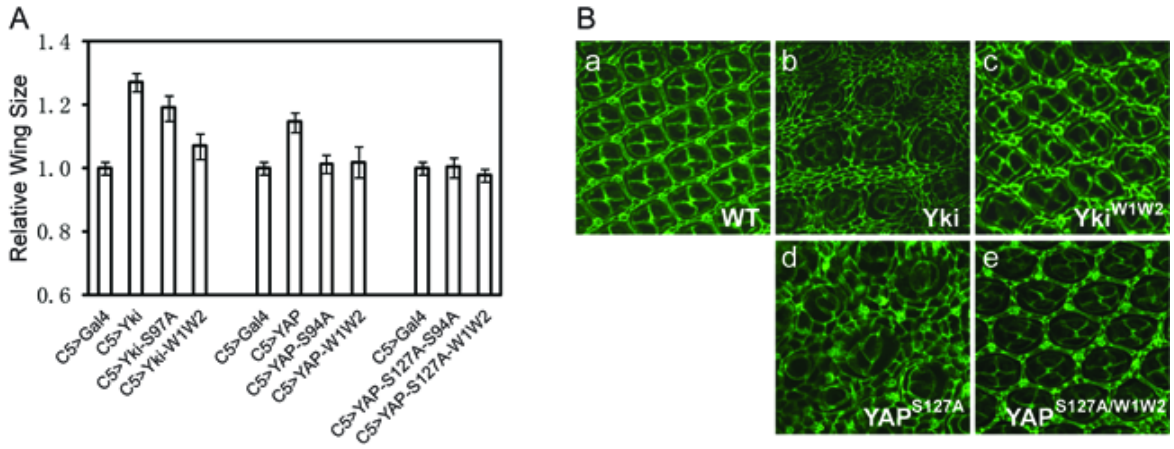


Figure 4.8 Supplemental to the WW domain plays a critical role in YAP/Yki induced tissue growth.

A. Quantification of wing sizes of flies overexpressing various *yki* and *YAP* genes.

B. Mutation of the WW domains of Yki and YAP significantly reduced their ability in inducing extra interommatidial cells in the developing eye. Cells in mid-pupal eye discs were outlined by Discs large (Dlg) antibody staining. The genotypes of the fly tissues are wild-type (Canton S) (panel *a*), *GMR-Gal4/UAS-yki-V5* (panel *b*), *GMR-Gal4/UAS-yki*^{W1W2}*-V5* (panel *c*), *GMR-Gal4/UAS-Flag-YAP*^{S127A} (panel *d*), and *GMR-Gal4/UAS-Flag-YAP*$^{S127A/W1W2}$ (panel *e*).

Discussion

YAP is a candidate oncogene which also regulates organ size. However, the mechanism by which YAP regulates oncogenesis and organ size is not well understood. Recent studies have demonstrated that the TEAD family transcription factors play a critical role in mediating YAP-dependent gene induction, growth promotion and transformation (Zhao et al. 2008). However, we also observed that a subset of YAP target genes could be induced by the TEAD-binding-defective YAP-S94A mutant (Zhao et al. 2008). Furthermore, *Drosophila* genetics study also showed that *yki* mutant cells have more severe growth defects than *sd* mutant cells (Paumard-Rigal et al. 1998; Simmonds et al. 1998; Huang et al. 2005), and overexpression of the Sd-binding-defective Yki-S97A elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (Zhao et al. 2008). 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 (Hao et al. 2007; Oka et al. 2008), 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 papers 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 (Hao et al. 2007), 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 wild type YAP enhances proliferation rate and confers serum-independent growth in

NIH-3T3 cells. The phosphorylation-defective YAP-5SA, but not the wild type 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.4.7D, we propose that under negative regulation by the Hippo pathway, YAP/Yki interact 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 (Yagi et al. 1999; Basu et al. 2003; Komuro et al. 2003), 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 (unpublished observation). All three RUNX family members have a conserved PPXY motif (Yagi et al. 1999). Efforts to simultaneously knockdown 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 fail to support serum independent growth, but rather promote 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 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.4.1C). 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 charactering 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 HXRXXS site, we found that YAP transformation potential is inhibited if serine 127 or 381 is intact. This result suggests that phosphorylation on 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 (Zhao et al. 2007). However, the mechanism by which phosphorylation of 381 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 pharmacological intervention in treating human cancer.

Contents of this chapter were published in the following paper: Zhao, B., Kim, J., Ye, X., Lai, Z.C., and Guan, K.L. 2008. Both TEAD binding and WW domains are required for the growth stimulation and oncogenic transformation activity of YAP. Cancer Res 69(3): 1089-1098.

Bibliography

Basu, S., Totty, N.F., Irwin, M.S., Sudol, M., and Downward, J. 2003. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Mol Cell* **11**(1): 11-23.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**(2): 401-415.

Camargo, F.D., Gokhale, S., Johnnidis, J.B., Fu, D., Bell, G.W., Jaenisch, R., and Brummelkamp, T.R. 2007. YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr Biol* **17**(23): 2054-2060.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. 2003. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* **30**(3): 256-268.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S.A., Gayyed, M.F., Anders, R.A., Maitra, A., and Pan, D. 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* **130**(6): 1120-1133.

Edgar, B.A. 2006. From cell structure to transcription: Hippo forges a new path. *Cell* **124**(2): 267-273.

Goulev, Y., Fauny, J.D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. 2008. SCALLOPED Interacts with YORKIE, the Nuclear Effector of the Hippo Tumor-Suppressor Pathway in Drosophila. *Curr Biol* **18**(6): 435-441.

Hanahan, D. and Weinberg, R.A. 2000. The hallmarks of cancer. *Cell* **100**(1): 57-70.

Hao, Y., Chun, A., Cheung, K., Rashidi, B., and Yang, X. 2007. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *J Biol Chem*.

Hariharan, I.K. 2006. Growth regulation: a beginning for the hippo pathway. *Curr Biol* **16**(24): R1037-1039.

Hariharan, I.K. and Bilder, D. 2006. Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. *Annu Rev Genet* **40**: 335-361.

Harvey, K. and Tapon, N. 2007. The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. *Nat Rev Cancer* **7**(3): 182-191.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. 2003. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**(4): 457-467.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**(3): 421-434.

Komuro, A., Nagai, M., Navin, N.E., and Sudol, M. 2003. WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. *J Biol Chem* **278**(35): 33334-33341.

Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., and Li, Y. 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120**(5): 675-685.

Modena, P., Lualdi, E., Facchinetti, F., Veltman, J., Reid, J.F., Minardi, S., Janssen, I., Giangaspero, F., Forni, M., Finocchiaro, G., Genitori, L., Giordano, F., Riccardi, R., Schoenmakers, E.F., Massimino, M., and Sozzi, G. 2006. Identification of tumor-specific molecular signatures in intracranial ependymoma and association with clinical characteristics. *J Clin Oncol* **24**(33): 5223-5233.

Oh, H. and Irvine, K.D. 2008. In vivo regulation of Yorkie phosphorylation and localization. *Development* **135**(6): 1081-1088.

Oka, T., Mazack, V., and Sudol, M. 2008. Mst2 and Lats kinases regulate apoptotic function of YAP. *J Biol Chem*.

Overholtzer, M., Zhang, J., Smolen, G.A., Muir, B., Li, W., Sgroi, D.C., Deng, C.X., Brugge, J.S., and Haber, D.A. 2006. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc Natl Acad Sci U S A* **103**(33): 12405-12410.

Paumard-Rigal, S., Zider, A., Vaudin, P., and Silber, J. 1998. Specific interactions between vestigial and scalloped are required to promote wing tissue proliferation in Drosophila melanogaster. *Dev Genes Evol* **208**(8): 440-446.

Saucedo, L.J. and Edgar, B.A. 2007. Filling out the Hippo pathway. *Nat Rev Mol Cell Biol* **8**(8): 613-621.

Simmonds, A.J., Liu, X., Soanes, K.H., Krause, H.M., Irvine, K.D., and Bell, J.B. 1998. Molecular interactions between Vestigial and Scalloped promote wing formation in Drosophila. *Genes Dev* **12**(24): 3815-3820.

Steinhardt, A.A., Gayyed, M.F., Klein, A.P., Dong, J., Maitra, A., Pan, D., Montgomery, E.A., and Anders, R.A. 2008. Expression of Yes-associated protein in common solid tumors. *Hum Pathol*.

Strano, S., Munarriz, E., Rossi, M., Castagnoli, L., Shaul, Y., Sacchi, A., Oren, M., Sudol, M., Cesareni, G., and Blandino, G. 2001. Physical interaction with Yes-associated protein enhances p73 transcriptional activity. *J Biol Chem* **276**(18): 15164-15173.

Sudol, M., Bork, P., Einbond, A., Kastury, K., Druck, T., Negrini, M., Huebner, K., and Lehman, D. 1995. Characterization of the mammalian YAP (Yes-associated

protein) gene and its role in defining a novel protein module, the WW domain. *J Biol Chem* **270**(24): 14733-14741.

Sudol, M. and Hunter, T. 2000. NeW wrinkles for an old domain. *Cell* **103**(7): 1001-1004.

Tao, W., Zhang, S., Turenchalk, G.S., Stewart, R.A., St John, M.A., Chen, W., and Xu, T. 1999. Human homologue of the Drosophila melanogaster lats tumour suppressor modulates CDC2 activity. *Nat Genet* **21**(2): 177-181.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. 2002. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**(4): 467-478.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat Cell Biol* **5**(10): 914-920.

Vassilev, A., Kaneko, K.J., Shu, H., Zhao, Y., and DePamphilis, M.L. 2001. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev* **15**(10): 1229-1241.

Wu, S., Huang, J., Dong, J., and Pan, D. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**(4): 445-456.

Wu, S., Liu, Y., Zheng, Y., Dong, J., and Pan, D. 2008. The TEAD/TEF Family Protein Scalloped Mediates Transcriptional Output of the Hippo Growth-Regulatory Pathway. *Dev Cell*.

Yagi, R., Chen, L.F., Shigesada, K., Murakami, Y., and Ito, Y. 1999. A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. *Embo J* **18**(9): 2551-2562.

Yokoyama, T., Osada, H., Murakami, H., Tatematsu, Y., Taniguchi, T., Kondo, Y., Yatabe, Y., Hasegawa, Y., Shimokata, K., Horio, Y., Hida, T., and Sekido, Y. 2008. YAP1 is involved in mesothelioma development and negatively regulated by Merlin through phosphorylation. *Carcinogenesis*.

Zender, L., Spector, M.S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S.T., Luk, J.M., Wigler, M., Hannon, G.J., Mu, D., Lucito, R., Powers, S., and Lowe, S.W. 2006. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* **125**(7): 1253-1267.

Zeng, Q. and Hong, W. 2008. The emerging role of the hippo pathway in cell contact inhibition, organ size control, and cancer development in mammals. *Cancer Cell* **13**(3): 188-192.

Zhang, J., Smolen, G.A., and Haber, D.A. 2008a. Negative regulation of YAP by LATS1 underscores evolutionary conservation of the Drosophila Hippo pathway. *Cancer Res* **68**(8): 2789-2794.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. 2008b. The TEAD/TEF Family of Transcription Factor Scalloped Mediates Hippo Signaling in Organ Size Control. *Dev Cell*.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.C., and Guan, K.L. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* **21**(21): 2747-2761.

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Lin, J.D., Wang, C.Y., Chinnaiyan, A.M., Lai, Z.C., and Guan, K.L. 2008. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* **22**(14): 1962-1971.

CHAPTER 5

CONCLUSION

The control of organ size is a basic biological question. In the last several years, the Hippo signaling pathway has been delineated and shown to be critical in control of organ size in both *Drosophila* and mammals. Acting downstream of the Hippo pathway is the Yki/YAP transcription co-activators. In mammalian cells, the Hippo pathway kinase cascade inhibits YAP by phosphorylation and promotion of their cytoplasmic localization. The TEAD family transcription factors have recently been identified as evolutionarily conserved key mediators of YAP biological functions. *yap* is a candidate oncogene, and several other components of the Hippo pathway are tumor suppressors. Dysregulation of the Hippo pathway contributes to the loss of contact inhibition observed in cancer cells. Therefore, the Hippo-YAP pathway connects the regulation of organ size and tumorigenesis.

Regulation of YAP phosphorylation and localization

The Hippo pathway induces Yki phosphorylation to control organ size in *Drosophila*. Regulation of such a basic biological process would be expected to be conserved in higher organisms. Indeed, YAP is directly phosphorylated by Lats on serine residues in five conserved HXRXXS motifs (Zhao et al. 2007; Hao et al. 2008), including S127 (Dong et al. 2007; Oka et al. 2008). Phosphorylation by Lats on this residue generates a 14-3-3 binding site and induces YAP cytoplasmic translocation, and therefore, inactivation (Zhao et al. 2007; Hao et al. 2008). Such a mechanism explains the Hippo

pathway-dependent nuclear/cytoplasmic translocation of YAP based on cell density. Consistently, keratinocytes lacking Hippo pathway component WW45 lost the cytoplasmic translocation of YAP upon Ca^{2+} induced differentiation (Lee et al. 2008). Removing the inhibitory phosphorylation sites disrupts the regulation of YAP localization and promotes YAP induced over-proliferation of NIH-3T3 cells (unpublished observation), oncogenic transformation of MCF10A cells (Hao et al. 2008), and overgrowth of *Drosophila* tissue *in vivo* (Zhao et al. 2007). In agreement with that, the transformation activity of YAP is inhibited by co-expression of Lats1 and Mst2 (Hao et al. 2008; Zhang et al. 2008a). These studies support the evolutionarily conserved function of YAP in promotion of cell proliferation and oncogenic transformation under negative regulation by the Hippo pathway.

YAP S127 has also been suggested to be an Akt phosphorylation site (Basu et al. 2003). However, the sequences around this site do not match the optimal Akt target site. YAP S127 phosphorylation is neither suppressed by PI3K inhibitors nor induced by EGF/ insulin stimulation or active Akt expression (Zhao et al. 2007). More importantly, YAP phosphorylation is not affected by knockout of PDK1, which is essential for Akt activity (Zhao et al. 2007). Consistent with that, the *Drosophila* Yki is not phosphorylated by Akt either (Dong et al. 2007). All these results strongly indicate that YAP is not directly phosphorylated by Akt at least under most physiological conditions. However, it cannot be excluded that YAP is phosphorylated by Akt under some circumstances.

Besides the Hippo pathway mediated serine/threonine phosphorylation, YAP was recently shown to be regulated by tyrosine phosphorylation. A recent report from Dr. Shaul's lab showed that c-Abl directly binds and phosphorylates YAP on Y357, which stabilizes YAP and confers selective binding of YAP to p73 and is required for cisplatin-induced apoptosis (Levy et al. 2008). In contrast with previously suggested mechanism of YAP-p73 activation involving Akt or RASSF, the Y357 phosphorylation and stabilization of YAP was shown to be indeed induced by DNA damage. However, the biochemical mechanism of Y357 phosphorylation in YAP activity regulation is not yet

clear, and it will be interesting to determine if there is any cross-talk between the Hippo pathway and c-Abl regulated YAP phosphorylation.

The Hippo pathway promotes YAP cytoplasmic retention. However, YAP does not have any obvious nuclear localization signal sequence. Therefore, it is not clear how YAP gets into the nucleus when the Hippo pathway is silenced. One possible mechanism is through interaction and co-transportation with target transcription factors, such as shown for *Drosophila* Yki, which is translocated from cytoplasm to nucleus by co-expression of Sd in S2 cells (Goulev et al. 2008; Zhang et al. 2008b). Such effect is overridden by the Hippo pathway as Hpo expression sequesters both Yki and Sd in the cytoplasm (Zhang et al. 2008b). More importantly, Sd expression significantly potentiates the effect of *wts* mutation in inducing Yki nuclear localization *in vivo* (Zhang et al. 2008b). Considering the functional conservation of Yki/Sd in the mammalian YAP/TEAD, such regulation likely exists for YAP, although it awaits confirmation. However, it has already been reported that upon cisplatin treatment, YAP translocates to the nucleus in a p73-dependent manner (Strano et al. 2005). It will be important to examine the contribution of different transcription factors in regulation of YAP nuclear localization and determine the underlying mechanism.

Transcription factor targets of YAP

As a transcription co-activator, YAP activates transcription by interacting with certain transcription factors. YAP binds to TEAD family transcription factors (Vassilev et al. 2001), which have four highly homologous proteins sharing a conserved DNA-binding TEA domain in human and mouse. Most adult tissues express at least one TEAD gene. YAP was first identified as a TEAD-interacting protein by affinity purification (Vassilev et al. 2001). Strikingly, about 75% of the purified TEAD2 are in complex with YAP. From a different direction, we screened for YAP targets in a Gal4-fusion transcription factor library, which covers about one third of potential transcription factors encoded by the human genome. This unbiased strategy identified TEAD2, TEAD3, and

TEAD4 as the strongest positives (Zhao et al. 2008). TEAD1, which is not in the library, is activated by YAP in similar magnitude. Therefore, biochemical purification starts with TEAD and functional screen starts with YAP complement each other nicely in establishing a partnership between YAP and TEAD at least in cell culture.

More importantly, TEAD was shown to play a critical role in YAP function. In MCF10A human mammary epithelial cells, YAP and TEAD1 promoter occupancy highly overlaps (Zhao et al. 2008). Knock-down of TEAD or introduction of a TEAD-binding-deficient mutation (serine 94 to alanine) in YAP aborts activation of a large fraction of YAP-inducible genes (Zhao et al. 2008). TEAD is further shown to be critical for YAP-induced overgrowth, epithelial-mesenchymal-transition (EMT), and oncogenic transformation in MCF10A cells (Zhao et al. 2008). Furthermore, the phenotype of TEAD1/TEAD2 double knockout mice resembles YAP knockout mice and evidence suggests that *tead1/tead2* and *yap* genetically interact with each other *in vivo* (Sawada et al. 2008). In addition, TEAD1/TEAD2 double knockout embryos show decreased proliferation and increased apoptosis (Sawada et al. 2008), a phenotype consistent with the Hippo pathway components mutants in *Drosophila*. Finally, the function of YAP and TEAD interaction in cell growth is implicated in human disease. Sveinsson's chorioretinal atrophy is a human genetic disease caused by a heterozygous mutation of a highly conserved tyrosine in the YAP binding domain of TEAD1 (Fossdal et al. 2004). Interestingly, mutation of this residue in TEADs abolished their interaction with and their activation by YAP (Kitagawa 2007; Zhao et al. 2008), which may explain the atrophic phenotype. These observations support that TEAD is downstream of the Hippo pathway mediating YAP activity.

However, we also observed that a subset of YAP target genes could be induced by the TEAD-binding-defective YAP-S94A mutant (Zhao et al. 2008). Furthermore, *Drosophila* genetics study also showed that *yki* mutant cells have more severe growth defects than *sd* mutant cells (Paumard-Rigal et al. 1998; Simmonds et al. 1998; Huang et al. 2005), and overexpression of the Sd-binding-defective Yki-S97A elicits a reduced but still obvious overgrowth in *Drosophila* eyes and wings (Zhao et al. 2008). 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. My data established the functional importance of YAP/Yki WW domains in gene expression induction, growth promotion, and oncogenic transformation.

YAP as an oncoprotein

YAP is a potent growth promoter. Overexpression of YAP increases organ size in *Drosophila* and saturation cell density in NIH-3T3 cell culture (Zhao et al. 2007). However, *yap* was termed a candidate oncogene only after it was shown to be in human chromosome 11q22 amplicon, which is detected in several human cancers (Overholtzer et al. 2006; Zender et al. 2006). Consistently, *yap* was shown to be amplified in human primary intracranial ependymomas by clinical study (Modena et al. 2006). Besides the genomic amplification, YAP expression and nuclear localization was also shown to be elevated in multiple types of human cancers (Zender et al. 2006; Dong et al. 2007; Zhao et al. 2007; Steinhardt et al. 2008). 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 (Overholtzer et al. 2006); YAP cooperates with *myc* oncogene to stimulate tumor growth in nude mice (Zender et al. 2006); and more interestingly, transgenic mice with liver-specific YAP overexpression show a dramatic increase in liver size and eventually develop tumors (Camargo et al. 2007; Dong et al. 2007). The above evidence strongly indicates the function of *yap* as an oncogene. However, YAP was also reported to be a tumor suppressor as its gene locus is deleted in some breast cancers with a correlated loss of YAP expression (Yuan et al. 2008). Further experiments such as conditional knockout animal model will finally clarify the role of YAP in tumorigenesis.

The oncogenic function of YAP is further supported by the tumor suppressor function of its inhibitory upstream Hippo pathway components. Lats1 knockout leads to

soft-tissue sarcoma and ovarian tumor development (St John et al. 1999). *mob*, an activating subunit of Lats, is mutated in both human and mouse cancer cells (Lai et al. 2005). Loss-of-function mutation of WW45 has been observed in several human cancer cell lines (Tapon et al. 2002). Furthermore, a recent report showed that knockout of *ww45* leads to hyperplasia and differentiation defects in mouse embryonic epithelial structures (Lee et al. 2008). Mer, which is further upstream of the Hippo pathway, is a well-established human tumor suppressor (Evans et al. 2000). Therefore, the Hippo pathway consists of many proven or candidate tumor suppressors that inhibit YAP oncoprotein.

Noteworthy, several studies showed a proapoptotic function of YAP, which was mainly explained by co-activation of p73 (Strano et al. 2005; Matallanas et al. 2007; Levy et al. 2008; Oka et al. 2008). So far, the proapoptotic activity of YAP was only observed by overexpression of YAP or in response to strong apoptotic stimuli, such as Fas activation or DNA damage. However, the effect of YAP overexpression *in vivo* was shown to be an increase of organ size and finally tumor formation without accompanied increase of apoptosis. In fact, YAP overexpression protects liver tissue from Fas induced apoptosis (Camargo et al. 2007; Dong et al. 2007). On the other hand, the *Drosophila* genetic studies have clearly established that Yki inhibits aopotosis *in vivo*. It is still possible that under certain conditions like DNA damage, YAP was tyrosine phosphorylated by c-Abl, which selectively activates YAP transcriptional activity on p73 to induce apoptosis.

Contact inhibition of cell growth, often referred to as a hallmark of cancer cells, has long been a mystery. However, the Hippo pathway may have opened the window a little bit to understand this phenomenon. Several components of this pathway have been implicated in contact inhibition. Mer becomes dephosphorylated and activated in confluent cells (Shaw et al. 1998; Morrison et al. 2001), which has been reported to be both necessary and sufficient for contact inhibition. Lats2 and WW45 are also related to contact inhibition as their knockout MEF cells show loss of contact inhibition (McPherson et al. 2004; Lee et al. 2008). Finally, YAP is phosphorylated and translocated to the cytoplasm by the Hippo pathway at high cell density in a Mer-

dependent manner (Zhao et al. 2007). More importantly, a dominant-negative form of YAP restores contact inhibition in ACHN (Zhao et al. 2007), a cancer cell line with activation of YAP due to WW45 mutation. These observations suggest a critical role of YAP and the Hippo pathway in contact inhibition. Indentifying the upstream signal of this pathway might solve a long-standing mystery in cell biology.

Key questions to be addressed

Genetic, cell biology, and biochemical studies have established the novel Hippo tumor suppressor pathway. Inhibition of YAP transcription co-activators is the major target of the Hippo pathway to regulate cell proliferation, apoptosis, and organ size in mammals (Zeng and Hong 2008). In spite of rapid progresses in the field, many key questions remain to be answered. Perhaps the most interesting question in the Hippo pathway is the upstream signals that activate the core components. The sensing of organ size *in vivo* and cell confluence *in vitro* are long-standing mysteries. It is reasonable to speculate that such a signal may act upstream of the Hippo pathway.

Equally important is what are the other transcription factors mediating the biological function of YAP. The PPXY-motif-containing transcription factors may interact with YAP WW domains, and are therefore possible candidates. A related question is how YAP and TAZ activate transcription. Although largely unknown, current evidence suggests mechanisms such as recruitment of histone modification factors or Mediator complex. Answering these questions is important in understanding the mechanism of YAP in control of cell growth and organ size.

In *Drosophila*, Yki activates expression of many genes, including *cycE*, *diap1* and *bantam* microRNA. However, in mammalian cells, cycE is not induced by YAP, and the *bantam* microRNA is not conserved, while induction of *birc5*, an IAP family member, is insufficient to explain the increased proliferation and organ size. CTGF is recently shown to be a direct YAP target gene important for YAP function in mammalian cells (Zhao et al. 2008). However, there is no evidence that CTGF homolog is an Yki target gene in

Drosophila. It would be very interesting if common genes in *Drosophila* and mammals mediate the Hippo pathway functions, especially, if there is a functional equivalent of the *bantam* microRNA in mammals.

In the next few years, one can expect exciting discoveries in the Hippo pathway. Advances in this field may not only solve the puzzle of size control and contact inhibition, but also provide new targets for treatment of human diseases such as atrophy and cancer.

Bibliography

Basu, S., Totty, N.F., Irwin, M.S., Sudol, M., and Downward, J. 2003. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Mol Cell* **11**(1): 11-23.

Camargo, F.D., Gokhale, S., Johnnidis, J.B., Fu, D., Bell, G.W., Jaenisch, R., and Brummelkamp, T.R. 2007. YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr Biol* **17**(23): 2054-2060.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S.A., Gayyed, M.F., Anders, R.A., Maitra, A., and Pan, D. 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* **130**(6): 1120-1133.

Evans, D.G., Sainio, M., and Baser, M.E. 2000. Neurofibromatosis type 2. *J Med Genet* **37**(12): 897-904.

Fossdal, R., Jonasson, F., Kristjansdottir, G.T., Kong, A., Stefansson, H., Gosh, S., Gulcher, J.R., and Stefansson, K. 2004. A novel TEAD1 mutation is the causative allele in Sveinsson's chorioretinal atrophy (helicoid peripapillary chorioretinal degeneration). *Hum Mol Genet* **13**(9): 975-981.

Goulev, Y., Fauny, J.D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. 2008. SCALLOPED Interacts with YORKIE, the Nuclear Effector of the Hippo Tumor-Suppressor Pathway in Drosophila. *Curr Biol* **18**(6): 435-441.

Hao, Y., Chun, A., Cheung, K., Rashidi, B., and Yang, X. 2008. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *J Biol Chem* **283**(9): 5496-5509.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**(3): 421-434.

Kitagawa, M. 2007. A Sveinsson's chorioretinal atrophy-associated missense mutation in mouse Tead1 affects its interaction with the co-factors YAP and TAZ. *Biochem Biophys Res Commun* **361**(4): 1022-1026.

Komuro, A., Nagai, M., Navin, N.E., and Sudol, M. 2003. WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. *J Biol Chem* **278**(35): 33334-33341.

Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., and Li, Y. 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120**(5): 675-685.

Lee, J.H., Kim, T.S., Yang, T.H., Koo, B.K., Oh, S.P., Lee, K.P., Oh, H.J., Lee, S.H., Kong, Y.Y., Kim, J.M., and Lim, D.S. 2008. A crucial role of WW45 in developing epithelial tissues in the mouse. *EMBO J* **27**(8): 1231-1242.

Levy, D., Adamovich, Y., Reuven, N., and Shaul, Y. 2008. Yap1 phosphorylation by c-Abl is a critical step in selective activation of proapoptotic genes in response to DNA damage. *Mol Cell* **29**(3): 350-361.

Matallanas, D., Romano, D., Yee, K., Meissl, K., Kucerova, L., Piazzolla, D., Baccarini, M., Vass, J.K., Kolch, W., and O'Neill, E. 2007. RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. *Mol Cell* **27**(6): 962-975.

McPherson, J.P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak-Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J., Kassam, F., Squire, J., Bruneau, B.G., Hande, M.P., and Hakem, R. 2004. Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. *Embo J* **23**(18): 3677-3688.

Modena, P., Lualdi, E., Facchinetti, F., Veltman, J., Reid, J.F., Minardi, S., Janssen, I., Giangaspero, F., Forni, M., Finocchiaro, G., Genitori, L., Giordano, F., Riccardi, R., Schoenmakers, E.F., Massimino, M., and Sozzi, G. 2006. Identification of tumor-specific molecular signatures in intracranial ependymoma and association with clinical characteristics. *J Clin Oncol* **24**(33): 5223-5233.

Morrison, H., Sherman, L.S., Legg, J., Banine, F., Isacke, C., Haipek, C.A., Gutmann, D.H., Ponta, H., and Herrlich, P. 2001. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* **15**(8): 968-980.

Oka, T., Mazack, V., and Sudol, M. 2008. Mst2 and Lats kinases regulate apoptotic function of YAP. *J Biol Chem*.

Overholtzer, M., Zhang, J., Smolen, G.A., Muir, B., Li, W., Sgroi, D.C., Deng, C.X., Brugge, J.S., and Haber, D.A. 2006. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proc Natl Acad Sci U S A* **103**(33): 12405-12410.

Paumard-Rigal, S., Zider, A., Vaudin, P., and Silber, J. 1998. Specific interactions between vestigial and scalloped are required to promote wing tissue proliferation in Drosophila melanogaster. *Dev Genes Evol* **208**(8): 440-446.

Sawada, A., Kiyonari, H., Ukita, K., Nishioka, N., Imuta, Y., and Sasaki, H. 2008. Redundant roles of Tead1 and Tead2 in notochord development and the regulation of cell proliferation and survival. *Mol Cell Biol* **28**(10): 3177-3189.

Shaw, R.J., McClatchey, A.I., and Jacks, T. 1998. Regulation of the neurofibromatosis type 2 tumor suppressor protein, merlin, by adhesion and growth arrest stimuli. *J Biol Chem* **273**(13): 7757-7764.

Simmonds, A.J., Liu, X., Soanes, K.H., Krause, H.M., Irvine, K.D., and Bell, J.B. 1998. Molecular interactions between Vestigial and Scalloped promote wing formation in Drosophila. *Genes Dev* **12**(24): 3815-3820.

St John, M.A., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M.L., Brownstein, D.G., Parlow, A.F., McGrath, J., and Xu, T. 1999. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat Genet* **21**(2): 182-186.

Steinhardt, A.A., Gayyed, M.F., Klein, A.P., Dong, J., Maitra, A., Pan, D., Montgomery, E.A., and Anders, R.A. 2008. Expression of Yes-associated protein in common solid tumors. *Hum Pathol*.

Strano, S., Monti, O., Pediconi, N., Baccarini, A., Fontemaggi, G., Lapi, E., Mantovani, F., Damalas, A., Citro, G., Sacchi, A., Del Sal, G., Levrero, M., and Blandino, G. 2005. The transcriptional coactivator Yes-associated protein drives p73 gene-target specificity in response to DNA Damage. *Mol Cell* **18**(4): 447-459.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. 2002. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**(4): 467-478.

Vassilev, A., Kaneko, K.J., Shu, H., Zhao, Y., and DePamphilis, M.L. 2001. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev* **15**(10): 1229-1241.

Yagi, R., Chen, L.F., Shigesada, K., Murakami, Y., and Ito, Y. 1999. A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. *Embo J* **18**(9): 2551-2562.

Yuan, M., Tomlinson, V., Lara, R., Holliday, D., Chelala, C., Harada, T., Gangeswaran, R., Manson-Bishop, C., Smith, P., Danovi, S.A., Pardo, O., Crook, T., Mein, C.A., Lemoine, N.R., Jones, L.J., and Basu, S. 2008. Yes-associated protein (YAP) functions as a tumor suppressor in breast. *Cell Death Differ*.

Zender, L., Spector, M.S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S.T., Luk, J.M., Wigler, M., Hannon, G.J., Mu, D., Lucito, R., Powers, S., and Lowe, S.W. 2006. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* **125**(7): 1253-1267.

Zeng, Q. and Hong, W. 2008. The emerging role of the hippo pathway in cell contact inhibition, organ size control, and cancer development in mammals. *Cancer Cell* **13**(3): 188-192.

Zhang, J., Smolen, G.A., and Haber, D.A. 2008a. Negative regulation of YAP by LATS1 underscores evolutionary conservation of the Drosophila Hippo pathway. *Cancer Res* **68**(8): 2789-2794.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. 2008b. The TEAD/TEF Family of Transcription Factor Scalloped Mediates Hippo Signaling in Organ Size Control. *Dev Cell*.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.C., and Guan, K.L. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* **21**(21): 2747-2761.

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Lin, J.D., Wang, C.Y., Chinnaiyan, A.M., Lai, Z.C., and Guan, K.L. 2008. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* **22**(14): 1962-1971.