Regulation of two WW domain-containing transcriptional co-regulators in mammalian cells

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Abstract

One of the major tasks in cancer research is to understand the regulation of transcription factors that are crucial in controlling cell proliferation, differentiation and apoptosis. To achieve this, transcriptional co-regulators and signaling pathways that control transcription factors have been extensively studied. One good example is the Runx family of proteins and two of their co-regulators, transcription co-activator with PDZ domain-binding motif (TAZ) and Yes-associated protein (YAP). TAZ and YAP are paralogous and serve as either co-activators or co-repressors depending on the cellular context. In an attempt to understand the underlying molecular mechanisms for this dual role in transcription regulation, and especially considering that Yorkie (a *Drosophila* homologue of YAP) is under control of the *hippo* kinase pathway, we considered the possibility that TAZ and YAP are the target of this pathway. As such, we sought to test the signaling pathways, including the *hippo*-like pathway, that regulate the function of TAZ and YAP in mammalian cells.

In this thesis research, I first set out to characterize how TAZ, in synergy with the histone acetyltranserase monocytic leukemia zinc finger protein (MOZ), activates Runx-dependent transcription in a signal-responsive manner. Then I used Runx-dependent transcription as a model system to study the regulation of TAZ. I found that both class I and II histone deacetylases interacted with TAZ and repressed its co-activator activity. In addition, TAZ is acetylated by and synergizes with CBP and p300, two paralogous acetyltransferases, to activate transcription. These results suggest that the activity of TAZ is subjected to regulation by acetylation and deacetylation. These results also

provide a molecular basis for the hypothesis that TAZ and YAP recruit or release co-repressors in response to cellular stimuli.

I then investigated the hippo-like pathway in mammalian cells. Excitingly, I found that the kinase large tumor suppressor 2 (LATS2) phosphorylates TAZ and YAP at multiple sites with a novel consensus sequence HXRXXS/T, where X is any residue. The phosphorylation led to 14-3-3-dependent cytoplasmic retention and transcriptional inactivation of TAZ and YAP. The cytoplasmic retention is stimulated by both LATS2 and upstream elements such as Mst1, the *hippo* orthologue in mammals, and WW45, a WW domain-containing protein that enhances the activity of Mst1, suggesting that upstream inputs are required to fully activate LATS2. Related to this, we observed dramatic nuclear export of YAP when cells are confluent, and this nuclear export is inhibited when the 14-3-3 binding site is mutated, indicating that cell-cell contact mediates full activation of the hippo-like pathway and leads to inactivation of YAP and perhaps TAZ by phosphorylation and nuclear export. These results suggest that the *Drosophila hippo* pathway is recapitulated in mammalian cells. Thus, I have identified and characterized a novel signaling pathway for TAZ and YAP, and have shown that cell confluency is an important signal to regulate the function of YAP and TAZ. In summary, this research has improved our understanding in both normal and aberrant transcriptional regulation, thereby shedding light on novel therapeutic strategies.

Résumé

Un des défis majeurs de la recherche sur le cancer est de comprendre la régulation de certains facteurs de transcription qui jouent un rôle clé dans le contrôle de la prolifération, de la différenciation et de l'apoptose. Pour cette raison, de nombreuses études approfondies sur les corégulateurs et les voies de signalisation ont été menées. Parmi celles-ci, la famille des protéines Runx et deux de leurs corégulateurs, TAZ (transcription co-activator with PD Z domain-binding motif) et YAP (Yes-associated protein) en sont un bon exemple. TAZ et YAP sont des protéines paralogues et agissent comme des coactivateurs ou des corepresseurs en fonction du contexte cellulaire. Puisque Yorkie, l'orthologue de YAP chez la drosophile, est contrôlé par la voie de signalisation de *hippo*, l'identification de celle qui régule les fonctions de TAZ et de YAP dans les cellules de Mammifères a été menée afin de comprendre les mécanismes moléculaires du double rôle de ces corégulateurs dans la régulation transcriptionnelle. Dans ce travail de thèse, la manière dont TAZ active la transcription dépendante de Runx, en synergie avec l'histone acétyltransférase MOZ (monocytic leukemia zinc finger protein) a été étudiée. Par la suite, la transcription médiée par Runx a été utilisée comme modèle afin d'étudier la régulation de TAZ. Il a ainsi été démontré que les histones deacétylases de classe I et II interagissent avec TAZ et répriment son activité de coactivateur dans la transcription dépendante de Runx. En outre, TAZ est acétylé par CBP/p300, deux acétyltransférases paralogues, et synergise avec ces deux protéines pour activer la transcription. Ces résultats suggèrent donc que l'activité de TAZ est régulée par un mécanisme d'acétylation et de déacétylation et que TAZ et YAP recrutent et relâchent des corépresseurs en réponse à différents stimuli cellulaires. À la lumière de ces informations,

la voie de signalisation *hippo*-like dans les cellules de Mammifères a été examinée. De manière intéressante, la kinase LATS2 (large tumor suppressor 2) phosphoryle TAZ et YAP sur plusieurs sites correspondant à une séquence consensus HXRXXS/T, où X représente n'importe quel acide aminé. Cette phosphorylation conduit à la rétention et à l'inactivation transcriptionnelle de TAZ de manière dépendante de la protéine 14-3-3. Cette rétention cytoplasmique de TAZ et de YAP est stimulée par LATS2 et par des molécules situées en amont telles que Mst1, l'orthologue de *hippo* chez les Mammifères, et WW45, une protéine contenant un domaine de liaison WW qui augmente l'activité de Mst1. Ces résultats suggèrent donc qu'outre la kinase LATS2, des protéines en amont sont nécessaires pour activer LATS2 de façon optimale. De plus, une exportation nucléaire de YAP est observée lorsque les cellules sont confluentes et cette exportation est abolie suite à la mutation du site de liaison de la protéine 14-3-3. Par conséquent, le contact cellulaire mène à une activation complète de la voie de signalisation *hippo*-like entrainant l'inactivation de YAP, et peut-être de TAZ, par phosphorylation et exportation nucléaire. Les résultats obtenus dans les cellules de Mammifères récapitulent ceux obtenus lors de l'étude de la voie de signalisation *hippo* chez la drosophile. D'une part, une nouvelle kinase de TAZ et YAP a été identifiée et caractérisée. D'autre part, la confluence cellulaire a été identifiée comme un signal important de régulation de la fonction de YAP. En résumé, cette étude a grandement amélioré la compréhension de la régulation transcriptionnelle normale et aberrante, mettant ainsi en lumière une stratégie thérapeutique potentielle.

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Preface

The Guidelines for Thesis preparation issued by The Faculty of Graduate Studies and Research at McGill University reads as follows:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

In addition to the manuscripts, the thesis must include the following: (a) a table of contents, (b) and abstract in English and French, (c) an introduction which clearly states the rational and objectives of the research, (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper), (e) a final conclusion and summary.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to

such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the coauthored papers. When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition, if not submitted previously."

According to these guidelines, I have chosen to write my thesis based on four manuscripts. The thesis is organized in seven chapters: (I) literature review; (II-V) research chapters with each having its own abstract, introduction, materials and methods, results, and discussion; (VI) general discussion; and (VII) contributions to original research.

Publications arising from this work

First-author manuscripts:

1. Xu, M. Pelletier, N., Goh, S.L., and Yang, X.J. (2007) Multiple histone deacetylases (HDACs) regulate Runx-dependent transcription in a signal-dependent manner. Manuscript in preparation

2. Xu, M., Xiao, L., Wang, K., Grégoire, S., Pelletier, N., and Yang, X.J. (2007) LATS2 regulates co-activator activities of WW domain containing proteins YAP and TAZ. Manuscript in preparation.

3. Xu, M., Wang, K,, Xiao, L. and Yang, X.J. (2007) Cell-cell contact regulates subcellular localization of WW domain containing proteins TAZ and YAP. Manuscript in preparation.

Other publications

1. Grégoire, S., Xiao, L., Nie, J., Zhang, X., Xu, M., Li, J., Wong, J., Seto, E. and Yang, X.J. (2007) Histone deacetylase 3 interacts with and deacetylates myocyte enhancer factor 2. *Mol. Cell. Biol.* **27**:1280-95.

2. Wang, K., Degerny, C., Xu, M. and Yang, X.J. (2008) YAP, TAZ and Yorkie: a family of transcriptional coregulators in signaling, development and disease. *Biochemitry and Cell Biology*, an invited review.

Contribution of other authors

Lin Xiao:

In Chapter 4 and 5, she engineered some constructs for YAP mutants which include F-YAP, F-YAP S127A, GFP-YAP S347A, and GFP-YAP 5SA.

Serge Grégoire:

In Chapter 4, he performed immunofluorescence with HeLa cells and supported my findings.

Nadine Pelletier

In Chapter 2, she provided some constructs. In Chapter 3, she helped to perform the *in vitro* acetylation assay of TAZ

Mukta Ullah

In Chapter 3, she performed the co-immunoprecipitation for the binding between Runx3 and HDACs.

Abbreviations

TAZ	Transcription co-activator with PDZ-binding domain
YAP	Yes-associated protein
MOZ	Monocytic leukemia zinc finger
MORF	MOZ-related factor
CBP	CREB-associated protein
Lats	Large tumor suppressor protein
HDAC	Histone deacetylase
HAT	Histone acetyltransferase
Fj	Four-joint
Mer	Merlin
Ex	Expanded
TBX5	T-box protein 5
GCN5	General control non-derepressible 5
TR	Thyroid receptor
IGF-1	Insulin-like growth factor-1
RXR	Retinoid X receptor
KPM	Kinase phosphorylated during mitosis
Yrk	Yorkie
Diap1	Drosophila inhibitor of apoptosis
MYST	MOZ, Ybf2/Sas3, Sas2 and Tip60
Wts	Warts
Mst1	Mammalian Sterile 20-like 1
PMA	Phorbol 12-myristate 13-acetate
GM-CSF	Granulocyte and monocyte-colony stimulating factor
CCD	Cleidocranial dysplasia
PDZ	PSD95, DlgA, ZO-1
PSD95	Post synaptic density protein
DlgA	Drosophila disk large tumor suppressor
ZO-1	Zonula occludens-1

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Chapter 1

Literature Review

1.1 Transcription factors and human diseases

Cancer, one of the leading causes of death worldwide, is characterized by uncontrolled cell proliferation, and arrested cell differentiation and/or apoptosis. During cell transformation, oncogene activation and tumor suppressor gene inactivation are the hallmark genotypes of cancer cells. Because of this, research attempting to understand cancer development has in part become the effort to understand the transcriptional and post-translational control of oncogenes and oncoproteins. Many of these oncogene products exhibit the properties of binding to certain DNA sequences and regulating mRNA synthesis. Such proteins are called transcription factors. Their mode of action in cancer development can be largely categorized into two classes. Some transcription factors, if ectopically expressed or amplified, exert their dominant effects in transforming cells. For example, Myc is able to induce various types of cancer when overexpressed, and is sometimes associated with poor prognosis of cancer patients (Kelly and Siebenlist, 1986; Pelengaris et al., 2002). Other transcription factors act mainly as tumor suppressors, the inactivation of which facilitates cancer development. This scenario is exemplified by the role of p53 in tumorigenesis. It is a master regulator of many cellular processes, including growth arrest, cellular senescence and apoptosis. p53 transcriptionally regulates several key players controlling cell growth such as p21, a major growth inhibitory protein and Bax, a pro-apoptotic protein (Yu and Zhang, 2005). Mutation of the p53 gene leads to uncontrolled cell growth and impairs apoptosis, resulting in cell transformation. So p53, the guardian of the cells, is the convergent point of many hazardous stimuli that pose threats to cells in terms of cellular transformation.

The importance of transcription factors in human disease is not limited to cancer. To avoid an exhaustive review, bone diseases will be used as examples. In the late 1990s, researchers cloned a key factor called Cbfa1, which is extremely important in bone development. Furthermore, mutation of the Cbfa1 gene was found to be responsible for cleidocranial dysplasia (CCD) (Lee et al., 1997; Mundlos et al., 1997; Otto et al., 1997). CCD is also known as cleidocranial dysostosis. It is a hereditary disease with variable penetrance. The affected patients exhibit skull, teeth and clavicle abnormalities. Other bones may also be affected such as pelvic bones, ribs and hand bones. While homozygous loss of the Cbfa1 gene leads to severe skeletal defects, heterozygous mice showed abnormalities typical of human CCD. Indeed, the deletion, insertion and missense mutation of the Cbfa1 gene were found in CCD patients (Lee et al., 1997). The mutations are not only present in the DNA binding domain of Cbfa1 causing loss of DNA binding capacity, but also present in the transactivation domain. Consistent with these findings, Y. Ito's group screened six Japanese CCD patients and identified Cbfa1 mutation in each of these patients (Zhang et al., 2000). In addition to Cbfa1, many other transcription factors with causative roles in several bone diseases (Yang and Karsenty, 2002).

The importance of transcription factors in normal development and human disease has attracted extensive attention. Among many of the efforts in understanding how transcription factors control physiological and pathological processes, the line of research focused on regulation of transcription factors by signaling pathways has become a major topic. In this chapter, I briefly introduce the evolution of the concepts about transcription

Table 1 List of bone diseases caused by deregulation of transcription factors.

Transcription factor	Characteristic motif	Affected cell type/function	Disease
Scleraxis	BHLH	Mesenchymal cells	ND
Sox9, -5, -6	HMG	Chondrocytes	Campomelic dysplasia
NFAT1	Rel	Chondrocytes	Cartilagious exostosis
ATF2	BZip	Chondrocytes	Hypochondroplasia
Cbfa1/Runx2	Runt	Osteoblasts	CCD
Osterix	Zinc-finger	Osteoblasts	ND
Cfos	BZip	Osteoclasts	Osteosarcoma, osteopetrosis
Pu.1	ETS	Osteoclasts	Osteopetrosis
NF-κB	Rel	Osteoclasts	Osteopetrosis
MITF	BHLH	Osteoclasts	Osteopetrosis
ΔFosB	Bzip	Osteoclasts	Osteosclerosis
Fra1	Bzip	Osteoclast	Osteosclerosis
Msx1	Hox	Patterning	Witkop syndrome
Msx2	Hox	Patterning	Craniosynostosis PFM1
Twist	BHLH	Osteoblasts	Saethre-Chotzen syndrome
Alx4	Hox	Patterning	PFM2
Dlx3	Hox	Patterning	Trichodontoosseous syndrome
Tbx3	T-box	Patterning	Ulnar-mammary syndrome
Tbx5	T-box	Patterning	Holt-Oram syndrome
Shox	Hox	Patterning	Dyschondrosteosis
HoxD13	Hox	Patterning	Synpolydactyly

Abbreviations: BHLH, basic helix-loop-helix; Bzip, basic leucine zipper; HMG, high-motility-group domain; MITF, microphthalmia transcription factor; ND, not determined PFM, parietal foramina (Yang and Karsenty, 2002).

factors and co-factors and then explore how signaling pathways regulate transcription factors and their co-factors.

1.2 A historic overview of how transcription factors were discovered

The discovery of transcription factors originated partially from the early work on Lambda (λ) phage. One stage of phage life cycle is called prophage. In this stage, the infected phage integrates its DNA into the genome of *E.coli* but fails to reproduce itself. In the meantime, the prophage prevents other phages from super-infection. In 1957, Kaiser and Jacob discovered that the prophage was maintained by the λ phage C1 gene product that, according to Jacob, should encode a repressor that represses the genes involved in the phage lytic cycle and immunity to other phages (Kaiser and Jacob, 1957). This transcription scheme fit well into the dominant notion in the early 1960s when Jacob and Monod, based on the information available, proposed a model in which the main theme in transcription control is about repressors: the gene should be spontaneously on unless a represser is present to switch it off and maintain a repressive state. In the years that followed, researchers attempted to understand the underlying mechanisms. In 1967, Mark Ptashne isolated the C1 gene product -- λ repressor from *E coli* (Ptashne, 1967). Later, it turned out that this repressor not only repressed its target genes, but also activated the same genes depending on its concentration (Ptashne, 1988; Ptashne et al., 1980). Therefore, λ repressor was also an activator. Structural analysis of λ repressor revealed that it inserts the α-helix into the major groove of the DNA double helix and uses the same surface to recruit Pol II. The concept of transcriptional activation was

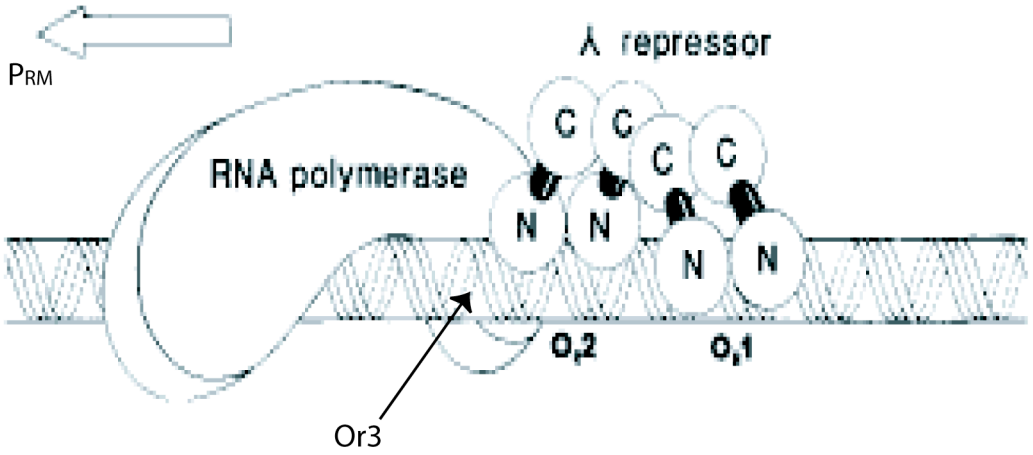

Figure 1 Molecular mechanism of transcriptional control by λ repressor. The repressor forms dimer and binds to the Or1 (right operator 1) site. The binding to the Or1 site facilitates to its binding to the Or2 site. The repressor cannot activate transcription until it recruits another dimer that binds to the Or2 site. The Or2-bound λ repressor uses the same surface (the N-terminal part), which binds to DNA, to recruit Pol II to activate the phage Pm gene. But if the repressor occupies the Or3 site alone, it represses promoter PRM that directs left side genes transcription (Adapted from Ptashne, 1988).

further exemplified by the studies on Gal4, which was found to induce transcription of a variety of genes in the reporter systems tested in yeast. Unlike λ repressor, however, Gal4 consists of two domains: a DNA-binding domain and an activation domain (Fischer et al., 1988; Ptashne, 2005; Sadowski et al., 1988). Thus, it seems that in eukaryotic cells, the structure of activators is more complicated than in prokaryotic cells. In eukaryotes, the DNA-binding domain and the activation domain are organized into two parts. The DNA-binding domain binds to the cognate DNA sequence while the activation domain recruits Pol II to activate transcription. Therefore, as shown above by the examples of λ repressor and Gal4, in both prokaryotic and eukaryotic cells, the repressive state of gene is not the singular mode; on the contrary, gene upregulation from their basal levels also confers important functions. As a result, new and more complete picture of the transcription world about how an activator works gradually evolved.

1.3 Runx proteins as examples

To date, thousands of transcription factors have been characterized in various organisms. Here, I use mammalian Runx transcription factors as examples to show how mammalian transcription factors work.

Runx transcription factors are mammalian homologues of the *Drosophila* Runt protein, which is crucial for fly segmentation (Gergen and Wieschaus, 1985). In mammals, this family consists of three members, each having unique functions: Runx1 is a key regulator in hematopoiesis and sensory neuron differentiation (Chen et al., 2006; Kramer et al., 2006; Zhong et al., 2006), Runx2 is important during osteogenesis (Ducy, 2000; Ducy and Karsenty, 1998; Ducy et al., 1999; Ducy et al., 1997; Harada et al., 1999;

Karsenty et al., 1999; Lee et al., 1997; Takeda et al., 2001), and Runx3 is crucial for gastric mucosa pathology (Li et al., 2002) and immune function (Woolf et al., 2003).

More importantly, all three members are involved in carcinogenesis and in some cases, cancer metastasis (Blyth et al., 2005; Ito, 2004). As a typical activator, Runx binds to the consensus DNA sequence, 5'-Py GPyGGTPy-3', through the Runt domain. One phenomenon is that many of the sequences similar to the consensus one can be found in the human genome using computational methods. However, in many cases Runx is unlikely to bind those sequences. Using DNA microarray technology, Kevin Struhl identified nearly 5800 p63 binding sites in the human genome, but only a fraction of them are responsive to p63 binding (Yang et al., 2006). This study suggested that an activator uses other means to achieve specific and stable DNA binding. Regarding Runx transcription factors, one strategy used by Runx is that they dimerize with CBFβ, a Runx binding partner that is also important in hematopoiesis, to achieve increased protein-DNA affinity and protein stability. The structure of the Runx-Cbfβ-DNA complex has been solved by nuclear magnetic resonance (NMR) and X-ray crystallography (Tahirov et al., 2001).

The DNA bound Runx proteins activate transcription through their activation domain. The activation domain was initially defined by virtue of its ability to activate reporter genes when transfected into cells regardless of the mechanisms. There are three such domains in Runx proteins (Fig. 2). To date, since there is no study to prove that Runx directly recruits Pol II, two major obstacles are in place for Runx to induce transcription: the distal position of many promoters and the structure of chromatin which is repressive for transcription. The looping mechanism is often utilized to bring the

promoter close to the transcription initiation site, thereby facilitating transcription. Overcoming the chromatin barrier, however, turns out to be far more complicated than first anticipated.

1.4 The discovery of transcription co-factors

In the early 1990s, several groups reported the identification and characterization of the activation domains in several nuclear receptor transcription factors (Barettino et al., 1994; Danielian et al., 1992; Durand et al., 1994). These studies were a major motive for the search of co-activator proteins because of the findings that different nuclear receptors interfered with each other when co-expressed. This observation suggested that a common pool of co-factors is used by several nuclear receptors to activate transcription. Researchers figured out that some activation domains of transcription factors recruit such co-factors that in turn contact the basal transcription machinery. Indeed, many co-activator proteins were soon discovered and the mechanisms for their activities were revealed (Glass et al., 1997).

Among these co-activators, CREB-binding protein (CBP) and p300 are of particular interest. CBP was cloned in 1993 for its ability to bind to phospho-CREB and activate CREB-dependent transcription (Chrivia et al., 1993). Later, CBP was shown to be a co-activator for several transcription factors (Chrivia et al., 1993; Kwok et al., 1994; Lundblad et al., 1995; Petrij et al., 1995). Its paralogue, p300, was also identified (Goodman and Smolik, 2000; Lundblad et al., 1995), but the mechanism for CBP/p300-mediated co-activation was unknown. Around the time, the Allis lab had long been working on histones and had uncovered a novel property of yeast GCN5 (general control

non-derepressible 5) (Brownell et al., 1996; Mizzen et al., 1996). The study showed that GCN5 possessed histone acetyltransferase (HAT) activity and proposed that the HAT activity is important for transcriptional activation. Consistent with this notion, another important finding was made that p300/CBP-associated factor (P/CAF), a GCN5 homologue in mammals, possesses HAT activity (Yang et al., 1996). Moreover, p300 and CBP were subsequently shown to possess intrinsic HAT activities (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). The research on these HATs rejuvenated the concept that histone acetylation is important for transcriptional activation, and added a new class of enzymes to the process of transription.

Histone acetylation was discovered in the 1960s and was speculated to be associated with gene activation (Allfrey et al., 1964; Nohara et al., 1966; Pogo et al., 1966). The proteins responsible for histone acetylation and the true biological significance of histone acetylation-mediated transcription activation had not been clear until the mid-1990s. The discovery of the HAT activities of GCN5, P/CAF, p300 and CBP provided strong evidence to support the notion that the transcription machinery recruits histone-modifying enzymes, including HATs, to activate gene transcription. Besides acetylation, histone can be methylated (Allfrey et al., 1964), phosphorylated (Jungmann and Schweppe, 1972; Louie and Dixon, 1972), ubiquitinated (Goldknopf et al., 1977), sumoylated (Shiio and Eisenman, 2003), ADP-ribosylated (Prieto-Soto et al., 1983) and also subjected to other modifications such as proline isomerization (Nelson et al., 2006). All these findings led to the proposal of several hypotheses explaining the consequences of multiple histone modification. Among them, the histone code hypothesis

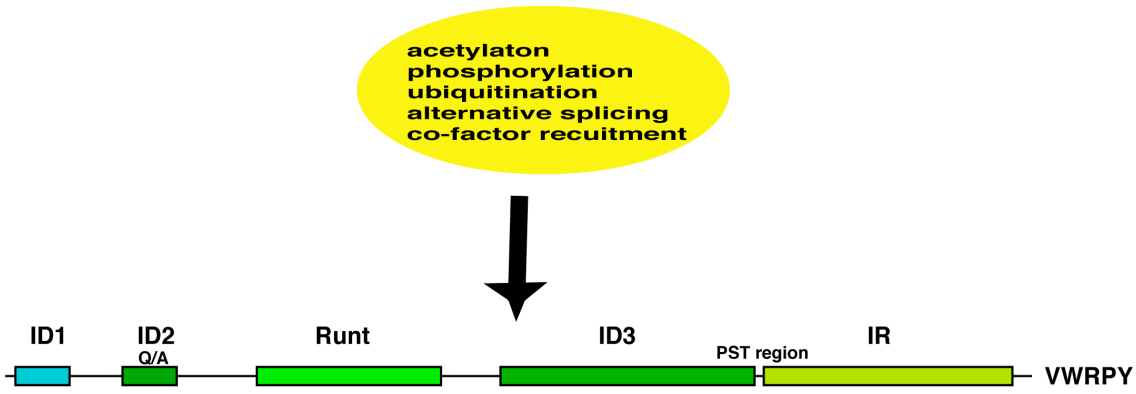

Figure 2 Domain structure of Runx2. IR, inhibitory domain; Q/A, Q and A rich domain; ID, activation domain. The activation or repression domain is denoted by their virtue of activating or repressing transcription using GAL-TK-Luc reporter.

has received wide attention (Jenuwein and Allis, 2001). It states that different histone modifications act individually or in combination to create docking sites to recruit different effectors, thus leading to different biological consequences. Now our knowledge about genetics should encompass both the genome and the epigenome, the knowledge of both is prerequisite to the ultimate understanding of the fidel translation of the genetic information into a living organism.

While researchers were working to identify and charaterize co-activator proteins, it became evident that many nuclear receptors could also repress transcription. For example, the ligand-binding domain of retinoic acid receptor (RAR) repressed transcription, and this repression domain was separable from the activation function-2 (AF-2) domain. This led to the purification of nuclear receptor co-repressor (N-CoR), silencing mediator for retinoid x receptor (RXR) and thyroid receptor (TR) (SMRT) (Horlein et al., 1995; Kurokawa et al., 1995). The repression domain interacts with these co-repressors to repress transcription.

1.5 Co-factors of Runx proteins

Many co-factors for Runx proteins have been identified. Co-repressors include Grouch/TLE (transducin-like enhancer of split) (Javed et al., 2000), mSin3a (Imai et al., 2004), histone deacetylases (Vega et al., 2004) and YAP (Zaidi et al., 2004). Co-activators include MOZ and MORF (Kitabayashi et al., 2001; Pelletier et al., 2002), TAZ (Cui et al., 2003), Rb (Thomas et al., 2001), and CBP/p300 (Sierra et al., 2003).

Among the co-repressors, histone deacetylases (HDACs) are a group of enzymes initially discovered over a decade ago for their ability to deacetylase histones (Rundlett et

al., 1996; Taunton et al., 1996; Wolffe, 1996). HDACs are often found as components of large co-repressor complexes and play crucial roles in transcription repression. Some non-histone proteins were also found to be substrates. Deacetylation of histone and non-histone proteins is extremely important biochemical reactions in the living organisms. The HDAC family is divided into four classes by analogy to yeast homologues (Yang and Gregoire, 2005). Class I members (HDAC1, 2, 3, 8 and 10), are homologous to yeast Rpd3, whereas class II members (HDAC4, 5, 6,7 and 9) are homologous to yeast Hda1 and contain an extended N-terminal part with important regulatory roles. Class III members (Sirt1-7) are homologous to yeast Sir2.

The importance of HDACs in regulating Runx-dependent transcription *in vivo* was shown in the HDAC4 knockout and transgenic mouse models (Vega et al., 2004). Both mouse models showed severe skeletal deformities, this phenotype was found to be due to abnormalities in chondrocyte development. HDAC4 knockout mice exhibited premature maturation of chondrocytes, which is reminiscent of the phenotype of Runx2 transgenic mice; HDAC4 overexpression, on the other hand, displayed delayed chondrocyte maturation. Subsequent biochemical studies confirmed that HDAC4 bound to Runx2 and repressed its transcriptional activity.

Among co-activators, the monocytic leukemia zinc finger protein (MOZ) belongs to the MYST family of histone acetyltransferases (HAT). The MOZ gene was identified as a fusion partner with the CBP gene (Borrow et al., 1996). Individuals harboring this fusion gene developed leukemia. MOZ is involved in several types of leukemia as shown by the fact that the MOZ gene is rearranged in t (8;16)(p11;p13) (Borrow et al., 1996) and inv(8)(p11q13) (Carapeti et al., 1998). Several other fusion genes, including MOZ-

p300 and MOZ-TIF2, have also been identified and both of them are involved in acute myeloid leukemia (Carapeti et al., 1998). As a co-activator, MOZ binds to Runx and activates Runx-dependent transcription. Aided by the recruitment of co-activators, the Runx transcription factor is able to modify chromatin structures through a complex enzymatic reaction. The exposed DNA provides an opportunity for transcription to start.

As exemplified by Runx transcription factors, many transcription factors are either activators or repressors, depending on how co-factors of different properties are recruited. In this sense, transcription factors themselves are reminiscent of adaptor proteins. Recruitment of one kind of protein and rejection of the other type by transcription factors seems to be dependent on the cellular context. Following this path, we are in a good position to raise the question if a co-activator can be a co-repressor in response to cell signaling. There is some evidence supporting this possibility. As discussed below, the examples are the transcription co-factors TAZ and YAP.

1.6 TAZ and YAP as transcriptional co-activators

TAZ (transcriptional co-activator with PD Z-binding domain) was first identified in affinity purification using immobilized 14-3-3 proteins (Kanai et al., 2000). The 14-3-3 binding site was mapped to serine 89. Sequence analysis revealed that TAZ contains a WW domain that binds to the PPXY motif. At the carboxyl terminus, TAZ has a PDZ (post-synaptic density protein PSD95, *Drosophila* tumor suppressor Dlg1, tight junction protein ZO-1) binding motif, suggesting that TAZ binds to PDZ domain-containing proteins. Indeed, studies have shown that TAZ interacts with NHERF-2, a PDZ protein that helps to connect cytoplasmic proteins to the actin cytoskeleton (Kanai et al., 2000).

The ability of WW domains to interact with PPXY motifs has led to the discovery of several proteins with the potential to interact with TAZ. These include Runx2 (Cui et al., 2003) and p73 (Strano et al., 2001) and putative ones like AP2, Krox20, Krox40, MEF2B, NF-E2, and Oct-4 (Kanai et al., 2000). Many of these proteins are transcription factors, suggesting that TAZ binds to these proteins and activates transcription. TAZ was also found to be a co-activator for TEF-1 (Mahoney et al., 2005), TBX5 (Murakami et al., 2005) and PAX3 (Murakami et al., 2006).

The sequence similarity between TAZ and YAP suggests analogous functional consequences. YAP was first identified as Yes kinase-associated protein (Sudol, 1994). Unlike TAZ, YAP possesses an SH3 binding domain and a proline-rich domain. Recent studies showed that the YAP gene was found in an amplicon on chromosome 11q22 (Overholtzer et al., 2006). The amplification of YAP is associated with proliferative advantages and cell transformation. More importantly, YAP expression is associated with an EMT (epithelial-mesenchymal transition) phenotype (Overholtzer et al., 2006) . But whether the oncogenic property of YAP is due to its transcriptional role is unclear. As expected, YAP is also a co-activator for several proteins such as p73 (Strano et al., 2001). YAP binds to p73 and activates p73-mediated transcription, suggesting that YAP possesses an intrinsic pro-apoptotic property. YAP has also been shown to be a co-activator of TEF-1 (transcriptional enhancer factor-1). Members of the TEF-1 family, which bind to MCAT (muscle C, A and T sites) and A/T-rich sites in promoters, are active in cardiac, skeletal and smooth muscle, placenta and neural crest (Chen et al., 1994; Mahoney et al., 2005),

1.7 Co-repressor activities of TAZ and YAP

Despite the established role of TAZ and YAP as potent co-activators for several transcription factors, there is some evidence suggesting that they are also co-repressors. An intriguing observation was made when TAZ was co-transfected with peroxisome proliferator-activated receptor γ (PPARγ) (Hong et al., 2005; Hong and Yaffe, 2006). PPARγ is a transcription factor that plays a crucial role in the control of differentiation from mesenchymal to adipose tissue. When co-transfected with TAZ, a decrease was observed in the transcriptional activity of PPARγ with or without ligand. This TAZ-mediated decrease in the transcriptional activity of PPARγ correlates well with the inhibitory role of TAZ in the PPARγ-driven differentiation of mesymchema into fat tissue. A similar observation has been made with YAP, which suppresses Runx2-dependent transcription in ROS17.18 cells (Hong and Yaffe, 2006; Westendorf, 2006; Zaidi et al., 2004). Although more evidence is needed to fully clarify the transcription inhibitory roles of TAZ and YAP, the concept that a co-activator can be a co-repressor and vice versa is emerging.

The fact that TAZ and YAP serve as either co-activators or co-repressors makes possible the conversion between these two distinct roles as well as the potential that signaling pathways might regulate this conversion. Accumulating evidence shows that the binding of co-factors to transcription factors is a regulated process: co-factors are either released or recruited when certain signaling pathways are activated. For example, CaMKIIΔ signaling releases mSin3a/HDAC co-repressor complex from MASH1 promoter, and recruits CBP/p300-containing co-activator complex (Ju et al., 2004). The question is whether or not such signaling pathways exist upstream of TAZ and YAP. So

far, studies indicate that TAZ and YAP are downstream elements of Akt and *hippo* signaling pathways.

1.8 The *hippo* pathway in *Drosophila* and mammals

1.8.1 Delineation of the *hippo* pathway in Drosophila for its role in regulating cell proliferation

Utilizing the FLP/FRT system (FLP is a recombinase in yeast and its target sequence is FRT site) (Fig. 3), Xu and colleagues screened *Drosophila* mutant mosaics in the early 1990s. The results led to the emergence of a new house keeping signaling pathway that both restricts proliferation and promotes apoptosis (Xu and Rubin, 1993; Xu et al., 1995). The first gene identified in this pathway is large tumor suppressor (LATS, also called Wats), the mutation of which resulted in tumor-like overgrowth in *Drosophila*. Later, *hippo*, sav and mob were isolated (Harvey et al., 2003; Hay and Guo, 2003; Kango-Singh et al., 2002; Lai et al., 2005; Pantalacci et al., 2003; Rothenberg and Jan, 2003; Ryoo and Steller, 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003). The mutation of any of these genes results in a similar overgrowth phenotype, suggesting that they interact genetically. Biochemical evidence supports that these proteins delineate a new pathway: with the recruitment of the WW domain-containing adaptor protein WW45, *hippo* phosphorylates and activates LATS, which is further activated by mob. Activated LATS phosphorylates and inactivates Yorkie (Huang et al., 2005).

Researchers are eagerly persuing the identity of the upstream inputs of this pathway. Onestudy demonstrated that *Drosophila* Merlin, a FERM domain adaptor protein that is homologous to mammalian NF2/Merlin (see below), is able to activate

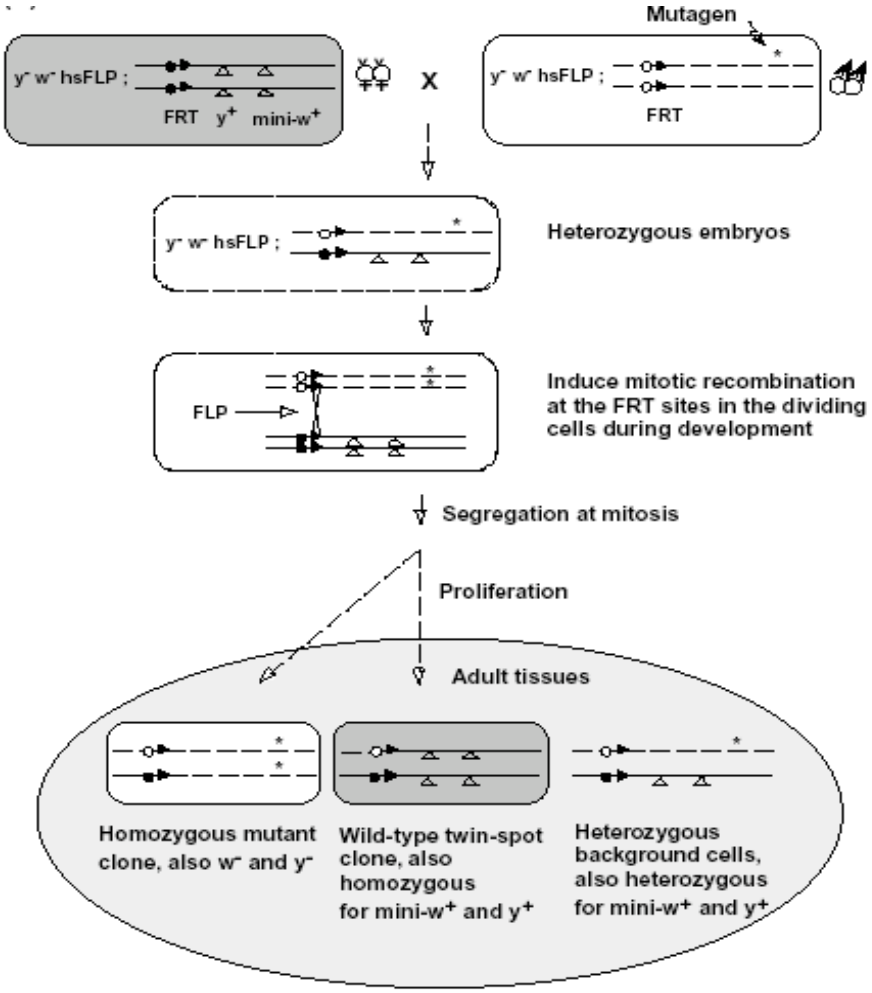

Figure 3 FLP/FRT system. The strain carrying FLP (hsFLP) and strain maker y (yellow) or (white) is introduced a mutation by X-ray irradiation. In the following embryo development, recombination at HRT site is induced allowing various recombinant daughter cells to be generated. The homozygous can be distinguished by its color (Adapted from Xu et al., 1995).

hippo. Mutation of Merlin leads to the overgrowth phenotype (Pellock et al., 2006). The research also revealed that Expaned (ex) is upstream of *hippo*. The Expanded gene earned its name because its mutation causes and expanded wing phenotype in fruit fly (Boedigheimer and Laughon, 1993). Although the mechanism behind Merlin/ex-mediated *hippo* activation remains unclear, the possibility of phosphorylation-related events by Merlin/ex can be ruled out because Merlin and Expanded are not kinases. The nature of Merlin and Expanded and the functions of this pathway make us favor the notion that upstream signals lie in cell-cell and cell-extracellular matrix communication, which is one subject of this thesis research.

Recent studies have added further insights to the understanding of the upstream signals. The research on *Drosophila* development made a surprising connection between the *hippo* and Fat pathways. The protocadherin protein Fat converges with the *hippo* pathway to restrict cell proliferation. The Fat gene was so-named duo to the fat phenotype (broad thorax and abdomen) of *Drosophila* homozygous of this mutant gene (Mahoney et al., 1991). It was also found that mutation of Fat caused cells in the imaginal disc of *Drosophila* to continue to grow to a final size much greater than its normal one (Bryant et al., 1988). Subsequently, the Fat gene was cloned in 1991. It belongs to the cadherin superfamily (Fig. 4) (Mahoney et al., 1991). Although the characterization of Fat is a recent event, work on the delineation of the Fat pathway dates back to 1919 when Bridge and Morgan described a molecule called Dachs. The mutation of Dachs resulted in defects in wing and leg growth. Recently, Dachs was found to be a downstream target of Fat (Mao et al., 2006). Fat binds to its ligand, Dachous (DS), another member of the procadherin family (Matakatsu and Blair, 2004), which leads to

inactivation of Dachs. The function of Fat is also influenced by Four-joint (fj), a mainly Golgi protein important in tissue polarity (Cho and Irvine, 2004). Strong evidence arises from the finding that mutation of both fj and DS leads to phenotypes similar to those observed in Dachs mutation (Cho and Irvine, 2004).

Besides its role in *Drosophila* development, Fat also acts like a tumor suppressor (Cho et al., 2006; Silva et al., 2006; Tyler and Baker, 2007; Tyler et al., 2007; Willecke et al., 2006), although the mechanisms behind this are not clear. Irvine's group made a significant breakthrough that further demonstrates the connection between the Fat and *hippo* pathways (Cho et al., 2006; Silva et al., 2006). They showed in vivo that Fat interacts genetically with the components of the *hippo* pathway. They also showed that Wts (Lats) physically interacts with Dachs, which provides an additional strong biochemical link between these two pathways.

1.8.2 The hippo-like pathway in mammals

"In the year of 1761, a man of threescore years of age came to St. Thomas' hospital…He had been accustomed during the greater part of his life to a constant succession of wens that shot out in several places of his head, trunk, arm and leg: which indisposition he inherited from his farther." This is a description by Dr. Mark Akenside of a case of neurofibromatosis (NF) (Ferner, 2007). The features are very typical. NF is comprised of heterogeneous disease entities divided into two categories: NF1 and NF2. NF1 is genetically distinct from NF2 and characterized by multiple and recurrent tumors

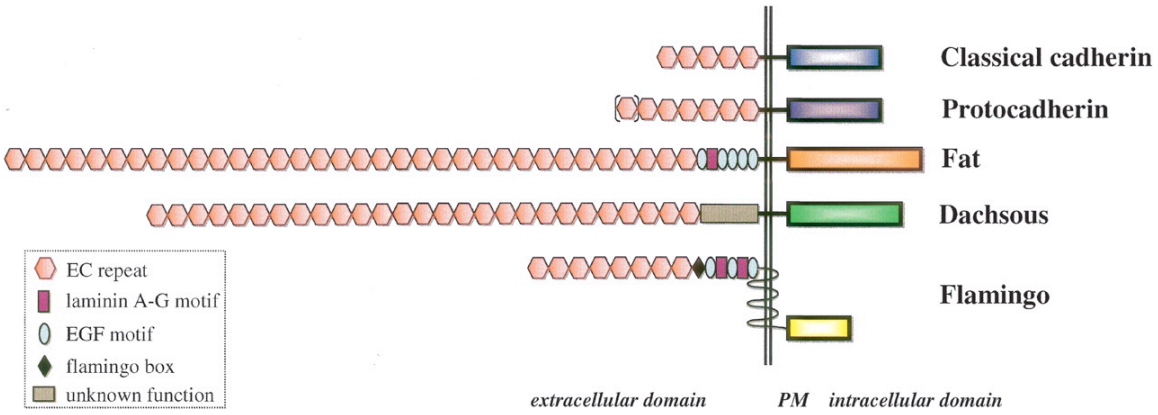

Figure 4 The Cadherin superfamily. All members of Cadherin family contain characteristic extracellular (EC) repeat. Non-classical members contain additional extracellular motifs. Flamingo is unique in that it has seven transmembrane domains (Adapted from Halbleib et al., 2006).

primarily in the central nervous system, the skin and the bone, while various other tissues can also be affected (Ferner, 2007).

The clinical symptoms of NF2 also include features of multiple organ tumor . The most affected organs are central nervous system, eye and skin. The incidence of the disease is about 1 to 235,000 with average onset of 18 to 24 years of age. Early genetic studies using DNA polymorphic makers located the gene for NF2 to chromosome 22 (Rouleau et al., 1990). In 1993, the NF2 gene was cloned (Rouleau et al., 1993; Trofatter et al., 1993). It has 17 exons coding a 595 amino acid protein known as Merlin. Merlin is highly conserved among different species. Since the discovery of Merlin, little has been added to the downstream elements. The recent studies in *Drosophila* have provided valuable clues that integrated Merlin into the *hippo* pathway in mammals (Fig. 5; Fig. 6).

1.8.3 LATS1 and LATS2/kpm as proteins for mitosis control and maintenance of genomic stability

The initial observation about the role of the *hippo* pathway in mitosis was made in *S. pombe*. In mammalian cells, immunofluorescence studies on LATS2 revealed its cytoplasmic and centrosomal localization. LATS2 was found to localize to the centrosome throughout the cell cycle (Hori et al., 2000). In M phase, LATS2 is phosphorylated by Aurora-A kinase to regulate γ-tubulin recruitment to the centrosome, suggesting that LATS2 plays a part in centrosome and mitotic spindle organization (Toji et al., 2004). Indeed, depletion of LATS2 results in impaired mitotic spindle organization. In vivo evidence emanated from LATS2 knockout animals. LATS2 knockout is

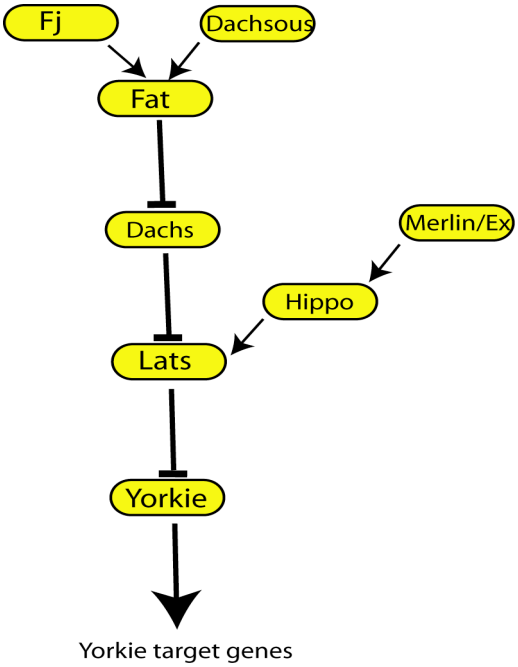

Figure 5 The model of integrated Fat and *hippo* pathways in *Drosophila*. Fat binds to its ligand Dachous to inhibit the function of Dachs, which poses an inhibitory role on Lats. Lats also receives activating signals from *hippo*. The net results of Fat and *hippo* stimulations are full activation of Lats.

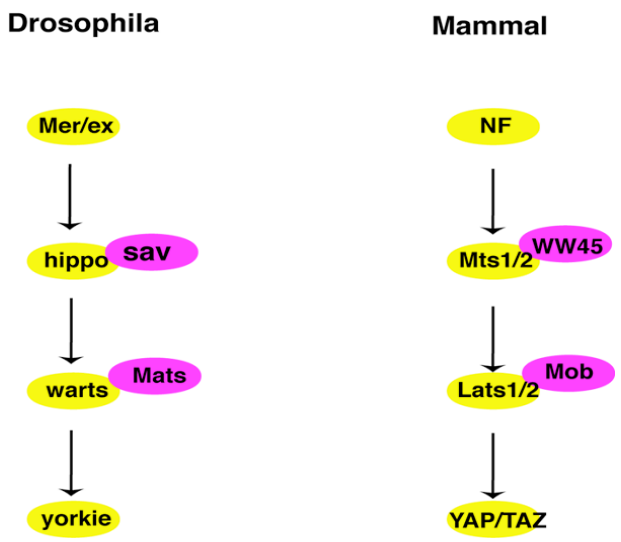

Figure 6 The components of the *hippo*-like pathway in *Drosophila* (left) and a similar pathway in mammals (right).

embryonic lethal, but MEFs derived from these animals displayed many profound defects in mitosis (McPherson et al., 2004). The LATS2$^{-/-}$ MEFs showed impaired cytokinesis, the last step of mitosis, which involves the successful cleavage of the midbody. LATS2$^{-/-}$ MEFs showed more frequent bridging between two daughter cells compared to the wild type, suggesting that the *hippo* pathway is crucial for cytokinesis. As expected, the LATS2$^{-/-}$ cells exhibited controsome amplification and multipolar mitotic spindles.

Besides LATS2, mammals possess a LATS2 paralogue: LATS1. The functions of LATS1 are similar to LATS2 in that both are important in cytokenesis (Yang et al., 2004) and cell cycle control (Xia et al., 2002). But differences are also obvious. LATS1 knockout mice are viable but develop soft tissue tumors at later stages. The mice also had pituitary dysfunction (St John et al., 1999). The reason that tumors develop only soft tissue in LATS knockout animals is unclear. But this seems contradict the fact that LATS1 expression starts early in embryonic development and is rather ubiquitous in various adult tissues (Tao et al., 1999). The possible explanation is functional redundancy of these two kinases. But tissue-specific functions of LATS1 are also a possible scenario, although there is no evidence to support this yet. Taken together, current evidence suggests that the *hippo* pathway regulates mitosis and maintains the genomic stability.

1.8.4 *Hippo* pathway is pro-apoptotic

Apoptosis is distinguished from necrotic cell death, in which local or systemic responses often occur. Apoptosis, on the other hand, is non-immflamatory. The process of apoptosis is achieved by the orchestration of various proteins and culminates in DNA fragmentation and apoptotic body formation. Apoptosis is extremely important in the development of

organisms. The development of the compound eye of *Drosophila* is a good example and has drawn extensive attention. The compound eye starts from the monolayer of epithelium lining the imaginal eye-antenna field. Later, an indented furrow called morphogenic furrow (MF) is formed within the field and "moves across". Before the MF, cells divide asynchronously, while cells behind it divide synchronously and form ommatidia, the individual units of the compound eye. The cells that are not in the ommatidia undergo a wave of apoptosis (Wolff and Ready, 1991). This apoptotic process has been elucidated (Harvey et al., 2003; Kamikubo et al., 2003; Pantalacci et al., 2003; Rothenberg and Jan, 2003; Ryoo and Steller, 2003; Udan et al., 2003; Wu et al., 2003). The *hippo* pathway is responsible for the cell death. The cells in the developing eye of *hippo* mutant *Drosophila* failed to undergo apoptosis. Later, *hippo* was shown to phosphorylate and inactivate Diap, a *Drosophila* apoptosis inhibitor. But the detailed mechanisms through which *hippo* induces apoptosis remain unclear.

Classical apoptosis of cells occurs mainly through two intertwined pathways. The extrinsic pathway involves cell signaling such as Fas. When Fas binds to its ligand, the death-inducing signaling complex (DISC) is recruited followed by caspases activation (Opferman, 2007). The intrinsic pathway mainly involves mitochondria. The death signals results in release of cytochrome c from the mitochondria, which ultimately activates caspases. The connection between *hippo*-induced apoptosis with this classical scheme remains largely uncharacterized. Aylon et al showed that a positive feedback loop exists between p53 and LATS2 (Aylon et al., 2006). LATS2 binds to MDM2 and inactivates its E3 ubiquitin ligase activity, which lead to p53 stabilization; in turn, p53 up-regulates LATS2 expression. This is a crucial finding in that it suggests that the pro-

apoptotic effect of the *hippo* pathway is, at least in part, mediated by p53. However, another line of research intriguingly indicated the existence of an alternative apoptosis pathway. Allis' group demonstrated that Ste20-like kinase Mst1 phosphorylates H2B at serine10, and this phosphorylation event is preceded by deacetylation of lysine 11in the same histone. H2B phosphorylation by Mst1 leads to DNA fragmentation and apoptosis (Ahn et al., 2006; Cheung et al., 2003). The question arising from these findings is whether or not a new apoptosis pathway exists. If so, the interplay between these pathways would be very interesting.

Subsequent research continues to reveal the pro-apoptotic role of the *hippo* pathway. Recent studies have linked *hippo* pathway to another important cell growth regulatory protein, Ras. It is well known that Ras expression in primary cells leads to cell senescence and cell death, which together comprise the main defense mechanisms of normal cells against oncoproteins (Leicht et al., 2007). Yet the reason for cell death in this process was not well understood. Discovery of Ras-associated proteins (RASSFs) provided a crucial link. It was found that RASSF1A mediates the apoptosis response in the presence of Ras (Dammann et al., 2000) and strikingly, RASSF1 was found to bind to Mst1 (Khokhlatchev et al., 2002). So the cellular transformation stress mediated by Ras activates the *hippo* pathway to counteract aberrant cell growth.

1.8.5 Yorkie, an ortholog of YAP, is downstream of the *hippo* pathway in *Drosophila*

The initial observation made about the *hippo* pathway was the cell-growth abnormalities when this signaling pathway is impaired. These cells exhibit not only overgrowth but also morphology changes (Justice et al., 1995). Although the reason for the altered

morphology remains unknown, significant progress has been made in understanding the mechanisms of this overgrowth. Deletion of the *hippo* pathway results in upregulation of several growth regulatory proteins. These include cyclin E, Diap1, an apoptosis inhibitor and Bantam, a microRNA that positively regulates cell growth (Nolo et al., 2006; Thompson and Cohen, 2006). An interesting question arising from this study is how down-regulation of the *hippo* pathway leads to up-regulation of those genes. The responsible protein remained unknown until the discovery of *Drosophila* Yorkie by Pan's group (Huang et al., 2005). Overexpression of Yorkie in *Drosophila* tissue results in overgrowth, which resembles phenotypes associated with mutation of others components of the *hippo* pathway. Later, Yorkie was shown by several groups to mediate its growth stimulatory role through upregulation of several genes: cyclin E, Diap1 and Bantam. Pan's group also showed that LATS2 phosphorylates and inactivates Yorkie. Since YAP is the mammalian homologue of Yorkie, it is possible that similar phenomena occur in mammals.

1.8.6 The fly *hippo* pathway plays a role in dendritic tiling

The *hippo* kinase is one of the two NDR family kinases in *Drosophila*. Emoto proved that the *hippo* pathway is involved in dendritic tiling and maintenance (Emoto et al., 2006). Dendrite tiling refers to the complete and non-overlaping coverage by dendrites of certain receptive areas. This process is controlled in part by the behavior of neurons. Studies indicate that dendrites avoid each other, and this 'like-repel-like' behavior seems to be

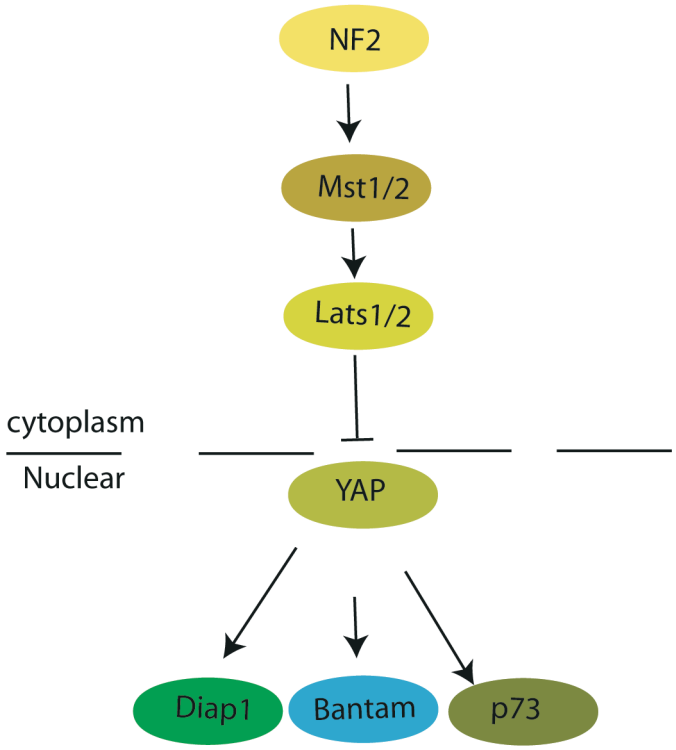

Figure 7 The *hippo*-like pathway in mammals. Bantam, a microRNA that positively regulates cell proliferation, is under transcription control of YAP; so is Diap1, an apoptosis inhibitor. YAP binds to p73 and activates its transcriptional activity.

controlled in part by a molecule called Flaminco. Mutation of Flaminco results in tiling abnormalities (Jan and Jan, 2003).

In Emoto's study, they showed that abnormalities in the *hippo* pathway resulted in defects in dendrite morphogenesis (Emoto et al., 2006). Neurons from Wts-negative flies showed hypobranching, less number of dendrite branches. Interestingly, besides hypobranching, neurons from *hippo*-negative flies displayed dendrite overlapping. Further, they showed that the dendrite formation defect occurred in a later stage rather than at the beginning. Taken together, these results convincingly establish the role of the *hippo* pathway in the development of neurons.

1.9 Rationale for the thesis projects

The intricately programmed transcriptional regulation is key to normal cell function, whereas deregulation results in malignant cell transformation. For example, both gain-of-function and loss-of-function of Runx proteins are often involved in cancer, especially Runx1, point mutations of the have been related to 5-10% AML (acute myelogenous leukemia) and MDS (myelodysplastic syndrome) (Hrusak et al., 1999; Hrusak et al., 1998; Trka et al., 1999; Trka et al., 1998; Zuna et al., 1999). The dominant oncogenic property of Runx proteins was shown by the CD2-Myc mice model, in which Runx2 gene is the frequent target of viral insertions in both the regulatory element and in the coding region (Ito, 2004). Viral insertions have also been identified in Runx1 and Runx3 genes (Blyth et al., 2001; Blyth et al., 2006; Ito, 2004). The crucial functions of Runx proteins in both development and tumorigenesis have led to extensive studies on the regulation of their transcriptional activities.

Our laboratory has been interested in the histone acetyltransferases MOZ and MORF (Yang, 2004). We have identified and characterized them as co-activators for Runx proteins (Pelletier et al., 2002). Beacuse the WW-domain protein TAZ was known to function as co-activators for Runx proteins (Cui et al., 2003), we investigated how MOZ and MORF interact with this WW-domain protein, and found that MOZ synergizes with TAZ to potently activate Runx-dependent transcription (Pelletier, N. & Yang, X.J., unpublished data). Interestingly, it was shown that TAZ and YAP can also be co-repressors (Hong et al., 2005; Hong and Yaffe, 2006; Westendorf, 2006; Zaidi et al., 2004). Moreover, the studies on the MSH1 promoter (Ju et al., 2004) provide the clue that these two distinct roles of TAZ and YAP could be subjected to cell signaling. We sought to identify signal pathways that could convert TAZ and YAP from co-activators into co-repressors, and vise versa. Five years ago, only the Akt pathway had been shown to phosphorylate and inactivate YAP (Basu et al., 2003), but this could not be reproduced (Goh, S.L. & Yang, X.J., unpublished data). In August 2005, it was reported that Yorkie, homologue of mammalian YAP, is in the *hippo* pathway of *Drosophila* (Huang et al., 2005), so we considered the exciting possibility whether the mammalian *hippo-like* pathway regulates the functions of TAZ and YAP.

Following this logical path, I carried out a series of studies, leading to four experimental chapters (i.e., Chapters 2-5). Chapter 2 provides a more detailed picture on how transcriptional co-activators such as MOZ and TAZ regulate Runx-dependent transcription and on how signaling pathways positively control this co-activation. Chapter 3, however, shows the opposite side of results that TAZ has the potentials to recruit co-repressors such as HDACs. Chapter 4 describes the signaling pathway

controlling TAZ and YAP, in which LATS2 appears to be the kinase phosphorylating and inactivating TAZ and YAP. More importantly, I have also found that HDACs are recruited to TAZ in the presence of the kinase, thereby demonstrating that LATS2 is the potential kinase that converts TAZ from a co-activator into a co-repressor. Chapter 5 adds an important observation that cell confluency is a crucial signaling input to activate LATS2 in mammalian cells. Together, this thesis provides novel molecular insights on transcription regulation by Runx proteins and other transcription factors, on the mechanisms of such regulation by transcriptional co-factors, and on how cellular signaling pathways control the function of these transcriptional co-factors.

1.10 References

Allfrey, V. G., Faulkner, R., and Mirsky, A. E. (1964). Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. Proc Natl Acad Sci U S A *51*, 786-794.

Bannister, A. J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. Nature *384*, 641-643.

Barettino, D., Vivanco Ruiz, M. M., and Stunnenberg, H. G. (1994). Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. Embo J *13*, 3039-3049.

Blyth, K., Cameron, E. R., and Neil, J. C. (2005). The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer *5*, 376-387.

Borrow, J., Stanton, V. P., Jr., Andresen, J. M., Becher, R., Behm, F. G., Chaganti, R. S., Civin, C. I., Disteche, C., Dube, I., Frischauf, A. M., *et al.* (1996). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nat Genet *14*, 33-41.

Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell *84*, 843-851.

Carapeti, M., Aguiar, R. C., Goldman, J. M., and Cross, N. C. (1998). A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. Blood *91*, 3127-3133.

Chen, C. L., Broom, D. C., Liu, Y., de Nooij, J. C., Li, Z., Cen, C., Samad, O. A., Jessell, T. M., Woolf, C. J., and Ma, Q. (2006). Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron *49*, 365-377.

Chen, Z., Friedrich, G. A., and Soriano, P. (1994). Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes Dev *8*, 2293-2301.

Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature *365*, 855-859.

Cui, C. B., Cooper, L. F., Yang, X., Karsenty, G., and Aukhil, I. (2003). Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. Mol Cell Biol *23*, 1004-1013.

Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. Embo J *11*, 1025-1033.

Ducy, P. (2000). Cbfa1: a molecular switch in osteoblast biology. Dev Dyn *219*, 461-471.

Ducy, P., and Karsenty, G. (1998). Genetic control of cell differentiation in the skeleton. Curr Opin Cell Biol *10*, 614-619.

Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M., and Karsenty, G. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev *13*, 1025-1036.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell *89*, 747-754.

Durand, B., Saunders, M., Gaudon, C., Roy, B., Losson, R., and Chambon, P. (1994). Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. Embo J *13*, 5370-5382.

Fischer, J. A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). GAL4 activates transcription in Drosophila. Nature *332*, 853-856.

Gergen, J. P., and Wieschaus, E. F. (1985). The localized requirements for a gene affecting segmentation in Drosophila: analysis of larvae mosaic for runt. Dev Biol *109*, 321-335.

Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997). Nuclear receptor coactivators. Curr Opin Cell Biol *9*, 222-232.

Goldknopf, I. L., French, M. F., Musso, R., and Busch, H. (1977). Presence of protein A24 in rat liver nucleosomes. Proc Natl Acad Sci U S A *74*, 5492-5495.

Goodman, R. H., and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev *14*, 1553-1577.

Harada, H., Tagashira, S., Fujiwara, M., Ogawa, S., Katsumata, T., Yamaguchi, A., Komori, T., and Nakatsuka, M. (1999). Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem *274*, 6972-6978.

Hong, J. H., Hwang, E. S., McManus, M. T., Amsterdam, A., Tian, Y., Kalmukova, R., Mueller, E., Benjamin, T., Spiegelman, B. M., Sharp, P. A.*, et al.* (2005). TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. Science *309*, 1074-1078.

Hong, J. H., and Yaffe, M. B. (2006). TAZ: a beta-catenin-like molecule that regulates mesenchymal stem cell differentiation. Cell Cycle *5*, 176-179.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature *377*, 397-404.

Imai, Y., Kurokawa, M., Yamaguchi, Y., Izutsu, K., Nitta, E., Mitani, K., Satake, M., Noda, T., Ito, Y., and Hirai, H. (2004). The corepressor mSin3A regulates phosphorylation-induced activation, intranuclear location, and stability of AML1. Mol Cell Biol *24*, 1033-1043.

Ito, Y. (2004). Oncogenic potential of the RUNX gene family: 'overview'. Oncogene *23*, 4198-4208.

Javed, A., Guo, B., Hiebert, S., Choi, J. Y., Green, J., Zhao, S. C., Osborne, M. A., Stifani, S., Stein, J. L., Lian, J. B., *et al.* (2000). Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. J Cell Sci *113 (Pt 12)*, 2221-2231.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science *293*, 1074-1080.

Ju, B. G., Solum, D., Song, E. J., Lee, K. J., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2004). Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway. Cell *119*, 815-829.

Jungmann, R. A., and Schweppe, J. S. (1972). Mechanism of action of gonadotropin. I. Evidence for gonadotropin-induced modifications of ovarian nuclear basic and acidic protein biosynthesis, phosphorylation, and acetylation. J Biol Chem *247*, 5535-5542.

Kaiser, A. D., and Jacob, F. (1957). Recombination between related temperate bacteriophages and the genetic control of immunity and prophage localization. Virology *4*, 509-521.

Kanai, F., Marignani, P. A., Sarbassova, D., Yagi, R., Hall, R. A., Donowitz, M., Hisaminato, A., Fujiwara, T., Ito, Y., Cantley, L. C., and Yaffe, M. B. (2000). TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. Embo J *19*, 6778-6791.

Karsenty, G., Ducy, P., Starbuck, M., Priemel, M., Shen, J., Geoffroy, V., and Amling, M. (1999). Cbfa1 as a regulator of osteoblast differentiation and function. Bone *25*, 107-108.

Kelly, K., and Siebenlist, U. (1986). The regulation and expression of c-myc in normal and malignant cells. Annu Rev Immunol *4*, 317-338.

Kitabayashi, I., Aikawa, Y., Nguyen, L. A., Yokoyama, A., and Ohki, M. (2001). Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. Embo J *20*, 7184-7196.

Kramer, I., Sigrist, M., de Nooij, J. C., Taniuchi, I., Jessell, T. M., and Arber, S. (2006). A role for Runx transcription factor signaling in dorsal root ganglion sensory neuron diversification. Neuron *49*, 379-393.

Kurokawa, R., Soderstrom, M., Horlein, A., Halachmi, S., Brown, M., Rosenfeld, M. G., and Glass, C. K. (1995). Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature *377*, 451-454.

Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R., and Goodman, R. H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature *370*, 223-226.

Lee, B., Thirunavukkarasu, K., Zhou, L., Pastore, L., Baldini, A., Hecht, J., Geoffroy, V., Ducy, P., and Karsenty, G. (1997). Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nat Genet *16*, 307-310.

Li, Q. L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X. Z., Lee, K. Y., Nomura, S., Lee, C. W., Han, S. B., *et al*. (2002). Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell *109*, 113-124.

Louie, A. J., and Dixon, G. H. (1972). Trout testis cells. II. Synthesis and phosphorylation of histones and protamines in different cell types. J Biol Chem *247*, 5498-5505.

Lundblad, J. R., Kwok, R. P., Laurance, M. E., Harter, M. L., and Goodman, R. H. (1995). Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. Nature *374*, 85-88.

Mahoney, W. M., Jr., Hong, J. H., Yaffe, M. B., and Farrance, I. K. (2005). The transcriptional co-activator TAZ interacts differentially with transcriptional enhancer factor-1 (TEF-1) family members. Biochem J *388*, 217-225.

Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., *et al.* (1996). The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. Cell *87*, 1261-1270.

Mundlos, S., Otto, F., Mundlos, C., Mulliken, J. B., Aylsworth, A. S., Albright, S., Lindhout, D., Cole, W. G., Henn, W., Knoll, J. H., *et al.* (1997). Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell *89*, 773-779.

Murakami, M., Nakagawa, M., Olson, E. N., and Nakagawa, O. (2005). A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. Proc Natl Acad Sci U S A *102*, 18034-18039.

Murakami, M., Tominaga, J., Makita, R., Uchijima, Y., Kurihara, Y., Nakagawa, O., Asano, T., and Kurihara, H. (2006). Transcriptional activity of Pax3 is co-activated by TAZ. Biochem Biophys Res Commun *339*, 533-539.

Nelson, C. J., Santos-Rosa, H., and Kouzarides, T. (2006). Proline isomerization of histone H3 regulates lysine methylation and gene expression. Cell *126*, 905-916.

Nohara, H., Takahashi, T., and Ogata, K. (1966). Acetylation of histones by pigeon liver enzymes. Biochim Biophys Acta *127*, 282-284.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953-959.

Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R., *et al.* (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell *89*, 765-771.

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*, 12405-12410.

Pelengaris, S., Khan, M., and Evan, G. (2002). c-MYC: more than just a matter of life and death. Nat Rev Cancer *2*, 764-776.

Pelletier, N., Champagne, N., Stifani, S., and Yang, X. J. (2002). MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. Oncogene *21*, 2729-2740.

Petrij, F., Giles, R. H., Dauwerse, H. G., Saris, J. J., Hennekam, R. C., Masuno, M., Tommerup, N., van Ommen, G. J., Goodman, R. H., Peters, D. J., and et al. (1995). Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. Nature *376*, 348-351.

Pogo, B. G., Allfrey, V. G., and Mirsky, A. E. (1966). RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. Proc Natl Acad Sci U S A *55*, 805-812.

Prieto-Soto, A., Gourlie, B., Miwa, M., Pigiet, V., Sugimura, T., Malik, N., and Smulson, M. (1983). Polyoma virus minichromosomes: poly ADP-ribosylation of associated chromatin proteins. J Virol *45*, 600-606.

Ptashne, M. (1967). ISOLATION OF THE lambda PHAGE REPRESSOR. Proc Natl Acad Sci U S A *57*, 306-313.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. Nature *335*, 683-689.

Ptashne, M. (2005). Regulation of transcription: from lambda to eukaryotes. Trends Biochem Sci *30*, 275-279.

Ptashne, M., Jeffrey, A., Johnson, A. D., Maurer, R., Meyer, B. J., Pabo, C. O., Roberts, T. M., and Sauer, R. T. (1980). How the lambda repressor and cro work. Cell *19*, 1-11.

Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci USA *93*, 14503-14508.

Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. Nature *335*, 563-564.

Shiio, Y., and Eisenman, R. N. (2003). Histone sumoylation is associated with transcriptional repression. Proc Natl Acad Sci U S A *100*, 13225-13230.

Sierra, J., Villagra, A., Paredes, R., Cruzat, F., Gutierrez, S., Javed, A., Arriagada, G., Olate, J., Imschenetzky, M., Van Wijnen, A. J., *et al.* (2003). Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the vitamin D3 receptor but not p300 intrinsic histone acetyltransferase activity. Mol Cell Biol *23*, 3339-3351.

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*, 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*, 2145-2152.

Tahirov, T. H., Inoue-Bungo, T., Morii, H., Fujikawa, A., Sasaki, M., Kimura, K., Shiina, M., Sato, K., Kumasaka, T., Yamamoto, M., *et al.* (2001). Structural analyses of DNA

recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFbeta. Cell *104*, 755-767.

Takeda, S., Bonnamy, J. P., Owen, M. J., Ducy, P., and Karsenty, G. (2001). Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev *15*, 467-481.

Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408-411.

Thomas, D. M., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell *8*, 303-316.

Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J. M., Richardson, J. A., *et al.* (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell *119*, 555-566.

Westendorf, J. J. (2006). Transcriptional co-repressors of Runx2. J Cell Biochem *98*, 54-64.

Wolffe, A. P. (1996). Histone deacetylase: a regulator of transcription. Science *272*, 371-372.

Woolf, E., Xiao, C., Fainaru, O., Lotem, J., Rosen, D., Negreanu, V., Bernstein, Y., Goldenberg, D., Brenner, O., Berke, G., *et al.* (2003). Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. Proc Natl Acad Sci U S A *100*, 7731-7736.

Yang, A., Zhu, Z., Kapranov, P., McKeon, F., Church, G. M., Gingeras, T. R., and Struhl, K. (2006). Relationships between p63 binding, DNA sequence, transcription activity, and biological function in human cells. Mol Cell *24*, 593-602.

Yang, X., and Karsenty, G. (2002). Transcription factors in bone: developmental and pathological aspects. Trends Mol Med *8*, 340-345.

Yang, X. J., and Gregoire, S. (2005). Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Mol Cell Biol *25*, 2873-2884.

Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature *382*, 319-324.

Yu, J., and Zhang, L. (2005). The transcriptional targets of p53 in apoptosis control. Biochem Biophys Res Commun *331*, 851-858.

Zaidi, S. K., Sullivan, A. J., Medina, R., Ito, Y., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. (2004). Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. Embo J *23*, 790-799.

Zhang, Y. W., Yasui, N., Kakazu, N., Abe, T., Takada, K., Imai, S., Sato, M., Nomura, S., Ochi, T., Okuzumi, S., *et al*. (2000). PEBP2alphaA/CBFA1 mutations in Japanese cleidocranial dysplasia patients. Gene *244*, 21-28.

Zhong, J., Pevny, L., and Snider, W. D. (2006). "Runx"ing towards sensory differentiation. Neuron *49*, 325-327.

Ahn, S. H., Diaz, R. L., Grunstein, M., and Allis, C. D. (2006). Histone H2B deacetylation at lysine 11 is required for yeast apoptosis induced by phosphorylation of H2B at serine 10. Mol Cell *24*, 211-220.

Aylon, Y., Michael, D., Shmueli, A., Yabuta, N., Nojima, H., and Oren, M. (2006). A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. Genes Dev *20*, 2687-2700.

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*, 11-23.

Blyth, K., Terry, A., Mackay, N., Vaillant, F., Bell, M., Cameron, E. R., Neil, J. C., and Stewart, M. (2001). Runx2: a novel oncogenic effector revealed by in vivo complementation and retroviral tagging. Oncogene *20*, 295-302.

Blyth, K., Vaillant, F., Hanlon, L., Mackay, N., Bell, M., Jenkins, A., Neil, J. C., and Cameron, E. R. (2006). Runx2 and MYC collaborate in lymphoma development by suppressing apoptotic and growth arrest pathways in vivo. Cancer Res *66*, 2195-2201.

Boedigheimer, M., and Laughon, A. (1993). Expanded: a gene involved in the control of cell proliferation in imaginal discs. Development *118*, 1291-1301.

Bryant, P. J., Huettner, B., Held, L. I., Jr., Ryerse, J., and Szidonya, J. (1988). Mutations at the fat locus interfere with cell proliferation control and epithelial morphogenesis in Drosophila. Dev Biol *129*, 541-554.

Cheung, W. L., Ajiro, K., Samejima, K., Kloc, M., Cheung, P., Mizzen, C. A., Beeser, A., Etkin, L. D., Chernoff, J., Earnshaw, W. C., and Allis, C. D. (2003). Apoptotic

phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. Cell *113*, 507-517.

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*, 1142-1150.

Cho, E., and Irvine, K. D. (2004). Action of fat, four-jointed, dachsous and dachs in distal-to-proximal wing signaling. Development *131*, 4489-4500.

Cui, C. B., Cooper, L. F., Yang, X., Karsenty, G., and Aukhil, I. (2003). Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. Mol Cell Biol *23*, 1004-1013.

Dammann, R., Li, C., Yoon, J. H., Chin, P. L., Bates, S., and Pfeifer, G. P. (2000). Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet *25*, 315-319.

Emoto, K., Parrish, J. Z., Jan, L. Y., and Jan, Y. N. (2006). The tumour suppressor Hippo acts with the NDR kinases in dendritic tiling and maintenance. Nature *443*, 210-213.

Ferner, R. E. (2007). Neurofibromatosis 1 and neurofibromatosis 2: a twenty first century perspective. Lancet Neurol *6*, 340-351.

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*, 457-467.

Hay, B. A., and Guo, M. (2003). Coupling cell growth, proliferation, and death. Hippo weighs in. Dev Cell *5*, 361-363.

Hong, J. H., Hwang, E. S., McManus, M. T., Amsterdam, A., Tian, Y., Kalmukova, R., Mueller, E., Benjamin, T., Spiegelman, B. M., Sharp, P. A., *et al.* (2005). TAZ, a

transcriptional modulator of mesenchymal stem cell differentiation. Science *309*, 1074-1078.

Hong, J. H., and Yaffe, M. B. (2006). TAZ: a beta-catenin-like molecule that regulates mesenchymal stem cell differentiation. Cell Cycle *5*, 176-179.

Hori, T., Takaori-Kondo, A., Kamikubo, Y., and Uchiyama, T. (2000). Molecular cloning of a novel human protein kinase, kpm, that is homologous to warts/lats, a Drosophila tumor suppressor. Oncogene *19*, 3101-3109.

Hrusak, O., Trka, J., Zuna, J., Bartunkova, J., and Stary, J. (1999). Are we ready to curtail testing for TEL/AML1 fusion? Pediatric Hematology Working Group in the Czech Republic. Leukemia *13*, 981-983.

Hrusak, O., Trka, J., Zuna, J., Houskova, J., Bartunkova, J., and Stary, J. (1998). Aberrant expression of KOR-SA3544 antigen in childhood acute lymphoblastic leukemia predicts TEL-AML1 negativity. The Pediatric Hematology Working Group in the Czech Republic. Leukemia *12*, 1064-1070.

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*, 421-434.

Ito, Y. (2004). Oncogenic potential of the RUNX gene family: 'overview'. Oncogene *23*, 4198-4208.

Jan, Y. N., and Jan, L. Y. (2003). The control of dendrite development. Neuron *40*, 229-242.

Ju, B. G., Solum, D., Song, E. J., Lee, K. J., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2004). Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor

complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway. Cell *119*, 815-829.

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*, 534-546.

Kamikubo, Y., Takaori-Kondo, A., Uchiyama, T., and Hori, T. (2003). Inhibition of cell growth by conditional expression of kpm, a human homologue of Drosophila warts/lats tumor suppressor. J Biol Chem *278*, 17609-17614.

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*, 5719-5730.

Khokhlatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X. F., Seed, B., and Avruch, J. (2002). Identification of a novel Ras-regulated proapoptotic pathway. Curr Biol *12*, 253-265.

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*, 675-685.

Leicht, D. T., Balan, V., Kaplun, A., Singh-Gupta, V., Kaplun, L., Dobson, M., and Tzivion, G. (2007). Raf kinases: Function, regulation and role in human cancer. Biochim Biophys Acta.

Mahoney, P. A., Weber, U., Onofrechuk, P., Biessmann, H., Bryant, P. J., and Goodman, C. S. (1991). The fat tumor suppressor gene in Drosophila encodes a novel member of the cadherin gene superfamily. Cell *67*, 853-868.

Mao, Y., Rauskolb, C., Cho, E., Hu, W. L., Hayter, H., Minihan, G., Katz, F. N., and Irvine, K. D. (2006). Dachs: an unconventional myosin that functions downstream of Fat to regulate growth, affinity and gene expression in Drosophila. Development *133*, 2539-2551.

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*, 3785-3794.

McPherson, J. P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak-Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J., *et al.* (2004). Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. Embo J *23*, 3677-3688.

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*, 1895-1904.

Opferman, J. T. (2007). Apoptosis in the development of the immune system. Cell Death Differ.

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

Pelletier, N., Champagne, N., Stifani, S., and Yang, X. J. (2002). MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. Oncogene *21*, 2729-2740.

Pellock, B. J., Buff, E., White, K., and Hariharan, I. K. (2006). The Drosophila tumor suppressors Expanded and Merlin differentially regulate cell cycle exit, apoptosis, and Wingless signaling. Dev Biol.

Rothenberg, M. E., and Jan, Y. N. (2003). Cell biology: the hippo hypothesis. Nature *425*, 469-470.

Rouleau, G. A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B., and et al. (1993). Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature *363*, 515-521.

Rouleau, G. A., Seizinger, B. R., Wertelecki, W., Haines, J. L., Superneau, D. W., Martuza, R. L., and Gusella, J. F. (1990). Flanking markers bracket the neurofibromatosis type 2 (NF2) gene on chromosome 22. Am J Hum Genet *46*, 323-328.

Ryoo, H. D., and Steller, H. (2003). Hippo and its mission for growth control. Nat Cell Biol *5*, 853-855.

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*, 2081-2089.

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*, 182-186.

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*, 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*, 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*, 767-774.

Toji, S., Yabuta, N., Hosomi, T., Nishihara, S., Kobayashi, T., Suzuki, S., Tamai, K., and Nojima, H. (2004). The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. Genes Cells *9*, 383-397.

Trka, J., Zuna, J., Haskovec, C., Brabencova, A., Kalinova, M., Muzikova, K., Paukertova, R., Hrusak, O., Zemanova, Z., Michalova, K., and Stary, J. (1999). [Detection of BCR/ABL, MLL/AF4 and TEL/AML1 hybrid genes and monitoring of minimal residual disease in pediatric patients with acute lymphoblastic leukemia]. Cas Lek Cesk *138*, 12-17.

Trka, J., Zuna, J., Hrusak, O., Kalinova, M., Muzikova, K., Lauschman, H., and Stary, J. (1998). Impact of TEL/AML1-positive patients on age distribution of childhood acute lymphoblastic leukemia in Czech Republic. Pediatric Hematology Working Group in Czech Republic. Leukemia *12*, 996-997.

Trofatter, J. A., MacCollin, M. M., Rutter, J. L., Murrell, J. R., Duyao, M. P., Parry, D. M., Eldridge, R., Kley, N., Menon, A. G., Pulaski, K., and et al. (1993). A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell *72*, 791-800.

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.

Tyler, D. M., Li, W., Zhuo, N., Pellock, B., and Baker, N. E. (2007). Genes affecting cell competition in Drosophila. Genetics *175*, 643-657.

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*, 914-920.

Westendorf, J. J. (2006). Transcriptional co-repressors of Runx2. J Cell Biochem *98*, 54-64.

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*, 2090-2100.

Wolff, T., and Ready, D. F. (1991). The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. Development *113*, 841-850.

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*, 445-456.

Xia, H., Qi, H., Li, Y., Pei, J., Barton, J., Blackstad, M., Xu, T., and Tao, W. (2002). LATS1 tumor suppressor regulates G2/M transition and apoptosis. Oncogene *21*, 1233-1241.

Xu, T., and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117*, 1223-1237.

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*, 1053-1063.

Yang, X., Yu, K., Hao, Y., Li, D. M., Stewart, R., Insogna, K. L., and Xu, T. (2004). LATS1 tumour suppressor affects cytokinesis by inhibiting LIMK1. Nat Cell Biol *6*, 609-617.

Yang, X. J. (2004). The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. Nucleic Acids Res *32*, 959-976.

Zaidi, S. K., Sullivan, A. J., Medina, R., Ito, Y., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. (2004). Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. Embo J *23*, 790-799.

Zuna, J., Hrusak, O., Kalinova, M., Muzikova, K., Stary, J., and Trka, J. (1999). TEL/AML1 positivity in childhood ALL: average or better prognosis? Czech Paediatric Haematology Working Group. Leukemia *13*, 22-24.

Chapter 2

TAZ synergizes with MOZ to activate Runx-dependent transcription in a signal-dependent manner

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Preface

One of the main themes in the literature review is on the co-factors and signals that govern the co-activator activities of TAZ and YAP. Our laboratory has been working on MOZ and MORF, and has demonstrated that they are potent co-activators for Runx2. As TAZ and YAP are also co-activators of Runx proteins, we sought to understand more details on how TAZ and MOZ could synergize to activate Runx-dependent transcription, and more importantly, on how signaling pathways control the activities of TAZ and MOZ on Runx-dependent transcription.

Abstract

The Runx family of transcription factors is crucial in many important developmental processes. These proteins are under tight control by multiple signaling pathways and co-factor recruitment, and their deregulation leads to cancer. In the past, much attention has been paid to the regulation of the Runx proteins themselves and, little is known about how their co-factors are regulated. MOZ and TAZ are previously identified co-activators for Runx. In our study, we further characterized the synergistic nature of these two independent co-factors in activation of Runx-dependent transcription. More importantly, we demonstrated that signaling pathways regulated the co-activators. Our co-immunoprecipitation study showed that signaling pathways such as PKC and MAPK induced the formation of a tight and stable co-activator complex, providing a molecular basis for target gene upregulation. Finally, we confirmed our finding by testing the endogenous GM-CSF level in HEK293 cells. In the presence of PMA, Runx2 alone induced about an 8-fold increase (2- to 3-fold without PMA) in GM-CSF level, and co-transfection with TAZ stimulated this effect further. In conclusion, our findings suggest that PKC and MAPK pathways modulate Runx-dependent transcription, and provide insights that signaling pathways also act through co-factors to regulate function Runx proteins.

Introduction

With a few exceptions, chromatin is generally repressive to transcription. The tight, ordered wrapping of DNA around nucleosomes represents the major barrier for transcription. In order for transcription to start in response to stimuli, the nucleosome has to be unwrapped by different mechanisms, one of which is a set of chromatin modifying enzymes. Exposed DNA is then accessible to transcription factors that in turn, recruit their co-factors to either activate or repress gene transcription. But this scenario just covers a few key steps. How each step happens is still poorly understood. One simple question is which comes first: DNA binding of transcription factors or unraveling of the chromatin. Current views favor transcription factor-initiated chromatin remodeling events, while signaling pathways can act directly on histones to change chromatin structure (Cheung et al., 2000; Mizzen et al., 1998). We have been working on the Runx family of transcription factors in an attempt to not only understand the regulation of these important proteins, but also to use them as examples to decipher the general picture of how transcription is controlled.

Runx family proteins consist of a group of Runt domain-containing transcription factors with key roles in osteogenesis (Ducy, 2000; Ducy and Karsenty, 1998; Ducy et al., 1999; Ducy et al., 1997), tumor development and progression (Stewart et al., 1997) (Ito, 2004), hematopoiesis, sensory neuron differentiation (Chen et al., 2006; Inoue et al., 2003; Inoue et al., 2002; Kramer et al., 2006; Marmigere et al., 2006; Yoshikawa et al., 2007) and muscle development (Wang et al., 2005). The diverse functions of Runx proteins necessitate tight control by multiple signaling pathways, including PTH (Selvamurugan et al., 2000), PKA (Boguslawski et al., 2000), TGFβ (Miyazono et al.,

2004), EGF (Xiao et al., 2002) and MAPK (Tanaka et al., 1996). Mechanistic studies have shown that signaling pathways induce elevated DNA-protein affinity and increased protein half life (Bae and Lee, 2006; Jin et al., 2004).

Runx proteins recruit co-repressors or co-activators to repress or activate transcription, respectively. The co-activators include Rb (Thomas et al., 2001), TAZ (Cui et al., 2003), MOZ and MORF (Kitabayashi et al., 2001a; Pelletier et al., 2002) and CBP/p300 (Sierra et al., 2003). Among them, TAZ (transcription co-activator with PDZ binding domain) is a WW domain-containing protein initially identified for its ability to bind 14-3-3 (Kanai et al., 2000). Later, TAZ was found to be a co-activator for T-box protein 5 (TBX5), a protein involved in Holt-Oram syndrome; homeodomain- containing protein PAX-3, the mutation of which results in Waardenburg syndrome and Runx proteins (Murakami et al., 2005; Murakami et al., 2006). Using its WW domain, TAZ binds to the PPXY motif of Runx and activates transcription.

Distinct from TAZ, MOZ belongs to the MYST family of histone acetyltransferases (Yang, 2004b). MOZ was identified in a fusion gene with CBP (Borrow et al., 1996a). Patients harboring this mutant gene develop leukemia. Several other fusion genes involving MOZ have been identified and nearly all of them are involved in blood disease (Troke et al., 2006). MOZ has also been shown to be the co-activator of Runx1 and Runx2 (Kitabayashi et al., 2001b). MOZ binds to Runx1 and Runx2 and activates Runx-dependent transcription potently. $MOZ^{-/-}$ mouse showed a defect in blood stem cells. (Katsumoto et al., 2006; Thomas et al., 2006). This further supports the notion that MOZ is an authentic player in hematopoiesis, mostly through

Runx1. Despite extensive functional studies, the mechanisms by which MOZ activates Runx-dependent transcription remain unexplored.

Although previous research on the regulation of Runx proteins by signaling pathways is abundant, little has been done to elucidate the impact of signaling pathways on the co-factors of Runx. Studies in this aspect would greatly improve our understanding of some general features of how a signaling pathway can affect transcription. In the present study, we provide evidence on how transcription co-factors work together to achieve efficient gene activation, shedding light on how signaling pathways control transcription.

Materials and methods

Mutagenesis of Runx1 — Long fidelity PCR-based mutagenesis was utilized to generate mutant Runx1 (Roche). The coding sequence of tyrosine (Y) was mutated into that for phenylalanine (F). The first PCR was performed using primers T7 (upstream of multiple cloning site of the pcDNA 3.1-based Runx1 expression vector) and 5' -GCG ACG AGC CGG GGG CGG GCG GCA GG- 3'. The second PCR was performed using primers 5' -CCT GCC CGC CCC CGG CTC GTC GC- 3' and HF2A, a primer downstream of Runx1 coding sequence. Products from the PCR were purified in low-melting agarose gels. Purified DNA was used as a template to amplify full length Runx1 by primers T7 and HF2A. Purified PCR product was ligated into pcDNA3.1 (-)-based expression vectors for HA- or Flag- tagged protein expression.

Protein-protein interaction — To examine the interaction between TAZ and MOZ proteins, using 20 μl SuperFect (Qiagen) for every 10 μg of DNA, the expression plasmids were co-transfected into HEK293 cells. Forty-eight hours after transfection, the cells were washed twice with PBS and collected in 0.5 ml of buffer K (20 mM sodium phosphate, pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, and protease inhibitors) containing 0.15 M KCl (buffer K150). Cell extracts were prepared for affinity purification on M2 agarose beads (Sigma). Beads with bound immunocomplex were washed four times with buffer K150, and bound proteins were eluted with Flag peptide (Sigma). Eluted proteins were subsequently resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to nitrocellulose membranes for Western analysis with either anti-Flag or anti-HA antibody. Blots were developed with Supersignal chemiluminescent substrates

(Pierce). For PMA treatment, 50 ng/ml of PMA was used. PMA dissolved in DMSO was added into the culture medium and incubated with cells for 18 h (or overnight).

Reporter gene assays — SuperFect transfection reagent (Qiagen) was used to transiently transfect luciferase reporter plasmids (100 to 400 ng) and/or mammalian expression plasmids (50 to 200 ng) into ROS17/2.8 or HEK293 cells. pBluescript KSII(+) (PKSII) was used to normalize the total amount of plasmids used in each transfection, and β-galactosidase (β-Gal) expression plasmid (50 ng) was co-transfected for normalization of transfection efficiency. After 18 h, cells were lysed, and luciferase reporter activity was determined by using D-(L)-luciferin (Boehringer Mannheim) as the substrate. Galactosidase activity was measured with Galacto-Light Plus (Tropix) as the substrate. The chemiluminescence from activated luciferin or Galacto-Light Plus was measured on a Luminometer plate reader (Dynex).

ELISA assays — HEK293 cells were transfected with the expression plasmids indicated. β-galactosidase expression plasmid was also transfected as an internal control. Total amount of plasmid was normalized by PKSII. 24 h after transfection, cells were treated with PMA as indicated. Cells were harvested and ELISA was performed according to manufacture's instructions (eBioscience or BD OptE1A™).

Results

TAZ and MOZ synergize to activate Runx-dependent transcription

To provide mechanistic insights on how Runx-dependent transcription is regulated by co-factors and signaling pathways, we set out to characterize known co-factors of Runx. Our previous study shows that MOZ and MORF activate Runx2-dependent transcription potently (Pelletier et al., 2002; Yang, 2004a). As TAZ is also a co-activator for Runx proteins (Cui et al., 2003), we investigated the outcomes when different co-activators are present. The results showed that TAZ and MOZ synergized to activate Runx1-dependent transcription, the potency of which was cell line-dependent (Fig. 1 and Supplementary figure (Pelletier, N., unpublished data)). In HEK293 cells, TAZ and MOZ caused a 60-fold increase of Runx1-dependent transcription, while less effect was observed in ROS17/2.8 cells (Fig. 1 and Supplementary figure (Pelletier, N., unpublished data)).

Runx2 has two transcriptional activation domains (Ducy, 2000), one of which contains a PPXY motif that is the recognition site for TAZ (Cui et al., 2003). The co-activation mediated by MOZ, however, is not through this PPXY motif since the Y358A mutant of Runx1 still uses MOZ as a co-activator (Fig. 1B, 1C). This suggests that TAZ and MOZ use distinct strategies to activate Runx-dependent transcription. More importantly, the synergistic effect is dependent on the PPXY motif in Runx1, suggesting that the co-activator activity of TAZ is necessary for the synergy (Fig. 1D).

The co-activation activities of TAZ and MOZ are responsive to PKC signaling

Previous studies have shown that the osteocalcin gene promoter possesses a responsive element to PMA (Boguslawski et al., 2000). Later, PMA was also shown to induce

phosphorylation of serines 276, 293, 303, 462 and threonine 300 of Runx1, which positively regulates Runx1-dependent transcription (Bae and Lee, 2006). PMA is known to activate PKC signaling pathway (Hermelin et al., 1988). We wanted to give further evidence that PKC can exert its effect on Runx proteins and their co-activators. To test this, we performed reporter gene assay using 6OSE2-luc reporter, which contains six Runx-binding sites of the osteocalcin promoter. The results demonstrated potent activation by PMA on Runx-dependent transcription on the 6OSE2-Luc reporter, and on the co-activation activities of TAZ (Fig. 2) and MOZ (data not shown). To reveal the direct link between the PKC pathway and PMA-mediated Runx activation, we used two inhibitors named GÖ6983 and GÖ6976 to block the PKC pathway. The results showed that the inhibitors could decrease or abolish the effect of PMA on Runx-dependent transcription (Fig. 2). Taken together, these data demonstrate that PKC signaling upregulates Runx-dependent transcription through Runx proteins and co-factors.

MAPK signaling pathway (MEK1) upregulates Runx-dependent transcription

Besides the PKC pathway, the MAPK signaling pathway has also been extensively studied and has been shown to upregulate transcriptional activity Runx proteins, although the results are controversial (Bae and Lee, 2006). A recent study showed *in vivo* that the MAPK pathway enhanced the transcription activities of Runx (Ge et al, 2007). Previously, we also found that a MEK inhibitor (UO126) partially inhibited PMA-induced increase in the transcriptional activity of Runx1 (Goh, S.L. & Yang, X.J., unpublished data). These findings suggest that the MAPK pathway is an important player in regulating Runx-dependent transcription. To characterize this further, we performed

reporter gene assays using the GM-CSF–Luc reporter in ROS17/2.8 cells, a rat osteosarcoma cell line. As shown in Fig. 3, our study demonstrated that MEK1dd, a constitutively active form of MEK1, was able to upregulate Runx-dependent transcription on GM-CSF-Luc reporter (Fig. 3). This data obtained in ROS17/2.8 cell further surports that MAPK signaling pathway upregulates Runx-dependent transcription.

PMA and MEK1 induce co-activator complex formation

Previous results (Pelletier, N., unpublished observation) showed that PMA treatment for 6 and 12 h dramatically increased the affinity between Runx2 and TAZ. Clearly, the upregulation of Runx2 activity upon PMA treatment was, at least in part, due to stronger co-activator binding. We next investigated whether PMA affected TAZ and MOZ interaction. We performed co-immunoprecipitation experiments by overexpressing TAZ and MOZ SM, a mutant of MOZ containing only the SM domain. We showed that TAZ bound to the MOZ SM domain, and this binding was significantly strengthened by PMA treatment and co-expression of MEK1dd (Fig. 4). PMA and MEK1 induce stronger co-activator association. This tightened protein interaction forms the molecular basis for the upregulation Runx-dependent transcription.

Runx2, TAZ complex activates endogenous GM-CSF

To determine the biological significance of our findings, we tested endogenous GM-CSF levels by ELISA upon reconstitution of Runx2, TAZ and MOZ complex by co-transfecting the expression plasmids into HEK293 cells. Without PMA treatment, Runx2 induced a 2- to 3- fold increase of GM-CSF level. As expected, TAZ mediated an

additional 30% increase. MOZ, however, did not show any effect (Fig. 5). Since PMA induced a potent increase in the transcriptional activities of Runx proteins (Fig. 2), we investigated if PMA could also cause an increase in endogenous GM-CSF levels in the presence of Runx2. Indeed, we observed a significant further increase in GM-CSF level by Runx2 in the presence of PMA (Fig. 5). We explain this as a result of enhanced endogenous co-factors recruitment, as suggested by our previous findings. To test this possibility, we co-transfected TAZ along with Runx2 in the presence of PMA. As expected, we observed that PMA strongly activates Runx2-dependent transcription in the presence of TAZ. However, MOZ did not show such activation even with PMA treatment (Fig. 5). Taken together, our preliminary results obtained in ELISA provide biological relevance for our previous findings.

Discussion

One of the major advances in understanding transcription control is the elucidation that an orchestra of multiple co-factors recruited by transcription factor is one of the major mechanisms regulating transcription. Those co-factors display different properties enabling the transcription factor to have different roles in transcription. Signaling pathways are the governing part to determine what occurs in terms of transcription activation or repression. Deciphering how co-factors work together and how signaling pathways control this process is of great significance in assisting us to understand the detailed picture of transcription. Our studies showed that the co-activator activities of TAZ and MOZ synergize to activate Runx-dependent transcription. We further demonstrated that this co-activation property is responsive to both PKC and MAPK signaling pathways. We also gave molecular insights of how this happened. A signaling-dependent co-activator complex was induced. The ELISA study demonstrated the role of transfected TAZ and MOZ on the levels of endogenous GM-CSF and yielded preliminary information on the consequences of these co-factors when encountering intact chromatin.

PMA treatment experiments not only gave us crucial clues that multiple signaling pathways are involved in controlling Runx-dependent transcription, but also gave rise to some unanticipated results. PMA stimulation facilitates Runx-dependent transcription and the co-activation by TAZ on chromatin template as shown in the ELISA study. The possible role of PMA in this process is in part due to a tightened co-activation complex by a phosphorylation event. The induction of at least 6 h is needed for the effect to be obvious (Pelletilier, unpublished data) indicating that some other events are occurring, such as induction of new protein expression. Another possible scenario is that, when a

transcription factor is present, it binds to its target DNA in chromatin with low affinity, if not at all, because of the inaccessibility of nucleosome DNA. Stable and tight binding awaits chromatin to be opened up either by the cell cycle or by histone phosphorylation which is mediated by signaling pathways (Cheung et al., 2000). PMA might play such roles although direct evidence is lacking.

The role of PMA may also be attributable to its role in cell cycle progression (Whitfield et al., 1973). More and more evidence is available to link cell cycle progression to gene expression. For example, HoxB gene expression under retinoic acid induction needs S phase progression (Fisher and Mechali, 2003). S phase is the genome-wide opening and decondensation of chromosomes and provides a window of opportunity for the transcription factors to bind. Once transcription factors bind to their target DNA, they recruit co-factors such as TAZ or MOZ to contact basic transcription machinery.

PMA treatment failed to induce the effect of MOZ, suggesting the distinct role of MOZ from TAZ. In the future, it is imperative to explore the mechanisms of how MOZ activates gene expression. MOZ is a histone acetyltransferases (HAT) (Borrow et al., 1996b; Hilfiker et al., 1997), but the HAT activity does not seem to be important for transcription activation in our assay system. MOZ also possesses a repression domain as shown in the reporter gene assay using Gal4-TK-Luc reporter. When full-length MOZ is fused to Gal4-DBD, there is no transcription activity (Kitabayashi et al., 2001a). But when part of the N-terminal part is truncated, an induction was observed. Besides, MOZ also has several uncharacterized domains (Champagne et al., 2001). To elucidate the

mechanisms of MOZ-mediated activation will surely improve our understanding of gene regulation and cancer development.

References

Bae, S. C., and Lee, Y. H. (2006). Phosphorylation, acetylation and ubiquitination: the molecular basis of RUNX regulation. Gene *366*, 58-66.

Boguslawski, G., Hale, L. V., Yu, X. P., Miles, R. R., Onyia, J. E., Santerre, R. F., and Chandrasekhar, S. (2000). Activation of osteocalcin transcription involves interaction of protein kinase A- and protein kinase C-dependent pathways. J Biol Chem *275*, 999-1006.

Borrow, J., Stanton, V. P., Jr., Andresen, J. M., Becher, R., Behm, F. G., Chaganti, R. S., Civin, C. I., Disteche, C., Dube, I., Frischauf, A. M., *et al.* (1996a). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nat Genet *14*, 33-41.

Borrow, J., Stanton, V. P., Jr., Andresen, J. M., Becher, R., Behm, F. G., Chaganti, R. S., Civin, C. I., Disteche, C., Dube, I., Frischauf, A. M., *et al.* (1996b). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nat Genet *14*, 33-41.

Champagne, N., Pelletier, N., and Yang, X. J. (2001). The monocytic leukemia zinc finger protein MOZ is a histone acetyltransferase. Oncogene *20*, 404-409.

Chen, C. L., Broom, D. C., Liu, Y., de Nooij, J. C., Li, Z., Cen, C., Samad, O. A., Jessell, T. M., Woolf, C. J., and Ma, Q. (2006). Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron *49*, 365-377.

Cheung, P., Allis, C. D., and Sassone-Corsi, P. (2000). Signaling to chromatin through histone modifications. Cell *103*, 263-271.

Cui, C. B., Cooper, L. F., Yang, X., Karsenty, G., and Aukhil, I. (2003). Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. Mol Cell Biol *23*, 1004-1013.

Ducy, P. (2000). Cbfa1: a molecular switch in osteoblast biology. Dev Dyn *219*, 461-471.

Ducy, P., and Karsenty, G. (1998). Genetic control of cell differentiation in the skeleton. Curr Opin Cell Biol *10*, 614-619.

Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M., and Karsenty, G. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev *13*, 1025-1036.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell *89*, 747-754.

Fisher, D., and Mechali, M. (2003). Vertebrate HoxB gene expression requires DNA replication. Embo J *22*, 3737-3748.

Hermelin, B., Cherqui, G., Bertrand, F., Wicek, D., Paul, A., Garcia, I., and Picard, J. (1988). Phorbol ester-induced protein kinase C translocation and lysosomal enzyme release in normal and cystic fibrosis fibroblasts. FEBS Lett *229*, 161-166.

Hilfiker, A., Hilfiker-Kleiner, D., Pannuti, A., and Lucchesi, J. C. (1997). mof, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in Drosophila. Embo J *16*, 2054-2060.

Inoue, K., Ozaki, S., Ito, K., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S. C., Yamashita, N., Itohara, S., Kudo, N., and Ito, Y. (2003). Runx3 is essential for the target-specific axon pathfinding of trkc-expressing dorsal root ganglion neurons. Blood Cells Mol Dis *30*, 157-160.

Inoue, K., Ozaki, S., Shiga, T., Ito, K., Masuda, T., Okado, N., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S. C., *et al*. (2002). Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat Neurosci *5*, 946-954.

Ito, Y. (2004). Oncogenic potential of the RUNX gene family: 'overview'. Oncogene *23*, 4198-4208.

Jin, Y. H., Jeon, E. J., Li, Q. L., Lee, Y. H., Choi, J. K., Kim, W. J., Lee, K. Y., and Bae, S. C. (2004). Transforming growth factor-beta stimulates p300-dependent RUNX3 acetylation, which inhibits ubiquitination-mediated degradation. J Biol Chem *279*, 29409-29417.

Kanai, F., Marignani, P. A., Sarbassova, D., Yagi, R., Hall, R. A., Donowitz, M., Hisaminato, A., Fujiwara, T., Ito, Y., Cantley, L. C., and Yaffe, M. B. (2000). TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. Embo J *19*, 6778-6791.

Katsumoto, T., Aikawa, Y., Iwama, A., Ueda, S., Ichikawa, H., Ochiya, T., and Kitabayashi, I. (2006). MOZ is essential for maintenance of hematopoietic stem cells. Genes Dev *20*, 1321-1330.

Kitabayashi, I., Aikawa, Y., Nguyen, L. A., Yokoyama, A., and Ohki, M. (2001a). Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. Embo J *20*, 7184-7196.

Kitabayashi, I., Aikawa, Y., Nguyen, L. A., Yokoyama, A., and Ohki, M. (2001b). Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. EMBO J *20*, 7184-7196.

Kramer, I., Sigrist, M., de Nooij, J. C., Taniuchi, I., Jessell, T. M., and Arber, S. (2006). A role for Runx transcription factor signaling in dorsal root ganglion sensory neuron diversification. Neuron *49*, 379-393.

Marmigere, F., Montelius, A., Wegner, M., Groner, Y., Reichardt, L. F., and Ernfors, P. (2006). The Runx1/AML1 transcription factor selectively regulates development and survival of TrkA nociceptive sensory neurons. Nat Neurosci *9*, 180-187.

Miyazono, K., Maeda, S., and Imamura, T. (2004). Coordinate regulation of cell growth and differentiation by TGF-beta superfamily and Runx proteins. Oncogene *23*, 4232-4237.

Mizzen, C., Kuo, M. H., Smith, E., Brownell, J., Zhou, J., Ohba, R., Wei, Y., Monaco, L., Sassone-Corsi, P., and Allis, C. D. (1998). Signaling to chromatin through histone modifications: how clear is the signal? Cold Spring Harb Symp Quant Biol *63*, 469-481.

Murakami, M., Nakagawa, M., Olson, E. N., and Nakagawa, O. (2005). A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. Proc Natl Acad Sci U S A *102*, 18034-18039.

Murakami, M., Tominaga, J., Makita, R., Uchijima, Y., Kurihara, Y., Nakagawa, O., Asano, T., and Kurihara, H. (2006). Transcriptional activity of Pax3 is co-activated by TAZ. Biochem Biophys Res Commun *339*, 533-539.

Pelletier, N., Champagne, N., Stifani, S., and Yang, X. J. (2002). MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. Oncogene *21*, 2729-2740.

Selvamurugan, N., Pulumati, M. R., Tyson, D. R., and Partridge, N. C. (2000). Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor alpha1. J Biol Chem *275*, 5037-5042.

Sierra, J., Villagra, A., Paredes, R., Cruzat, F., Gutierrez, S., Javed, A., Arriagada, G., Olate, J., Imschenetzky, M., Van Wijnen, A. J., *et al.* (2003). Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the vitamin D3 receptor but not p300 intrinsic histone acetyltransferase activity. Mol Cell Biol *23*, 3339-3351.

Stewart, M., Terry, A., Hu, M., O'Hara, M., Blyth, K., Baxter, E., Cameron, E., Onions, D. E., and Neil, J. C. (1997). Proviral insertions induce the expression of bone-specific isoforms of PEBP2alphaA (CBFA1): evidence for a new myc collaborating oncogene. Proc Natl Acad Sci U S A *94*, 8646-8651.

Tanaka, T., Kurokawa, M., Ueki, K., Tanaka, K., Imai, Y., Mitani, K., Okazaki, K., Sagata, N., Yazaki, Y., Shibata, Y., *et al.* (1996). The extracellular signal-regulated kinase pathway phosphorylates AML1, an acute myeloid leukemia gene product, and potentially regulates its transactivation ability. Mol Cell Biol *16*, 3967-3979.

Thomas, D. M., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell *8*, 303-316.

Thomas, T., Corcoran, L. M., Gugasyan, R., Dixon, M. P., Brodnicki, T., Nutt, S. L., Metcalf, D., and Voss, A. K. (2006). Monocytic leukemia zinc finger protein is essential for the development of long-term reconstituting hematopoietic stem cells. Genes Dev *20*, 1175-1186.

Troke, P. J., Kindle, K. B., Collins, H. M., and Heery, D. M. (2006). MOZ fusion proteins in acute myeloid leukaemia. Biochem Soc Symp, 23-39.

Wang, X., Blagden, C., Fan, J., Nowak, S. J., Taniuchi, I., Littman, D. R., and Burden, S. J. (2005). Runx1 prevents wasting, myofibrillar disorganization, and autophagy of skeletal muscle. Genes Dev *19*, 1715-1722.

Whitfield, J. F., MacManus, J. P., and Gillan, D. J. (1973). Calcium-dependent stimulation by a phorbol ester (PMA) of thymic lymphoblast DNA synthesis and proliferation. J Cell Physiol *82*, 151-156.

Xiao, G., Jiang, D., Gopalakrishnan, R., and Franceschi, R. T. (2002). Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. J Biol Chem *277*, 36181-36187.

Yang, X. J. (2004a). The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. Nucleic Acids Res *32*, 959-976.

Yang, X. J. (2004b). Lysine acetylation and the bromodomain: a new partnership for signaling. Bioessays *26*, 1076-1087.

Yoshikawa, M., Senzaki, K., Yokomizo, T., Takahashi, S., Ozaki, S., and Shiga, T. (2007). Runx1 selectively regulates cell fate specification and axonal projections of dorsal root ganglion neurons. Dev Biol *303*, 663-674.

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Supplementary figure

MOZ and TAZ are synergistic co-activators of Runx2

(A) Schematic representation of the luciferase reporters 6OSE2-Luc, OG2-Luc and GM-CSF-Luc. OG2-Luc contains endogenous mouse osteocalcin gene promoter region. It contains OSE1 and OSE2. 6OSE2-Luc was constructed by putting six identical OSE2 sequence together, and 6OSE2 was fused with Luc gene. GM-CSF-Luc contains the endogenous GM-CSF gene promoter.

(B-F) Luciferase reporters (400 ng) were transfected into HEK293, ROS17/2.8 or NIH3T3 cells along with an internal control plasmid (β-Gal, 5 ng) and expression plasmids for Runx2 (50 ng), Flag-MOZ (100 ng), Flag-TAZ (100 ng) and CBFβ_2 (200 ng) as indicated. Luciferase and β-galactosidase (β-Gal) activities were measured.

Figure 1

TAZ and MOZ synergize to activate Runx-dependent transcription in a PPXY motif-dependent manner

(A) Both TAZ and MOZ activate Runx1-dependent transcription, and their co-activator activities synergize. GM-CSF-Luc reporter plasmid was transfected into HEK293 along with the other plasmids for indicated proteins. β-Gal plasmid was also transfected as an internal control, 24 h after transfection, cells were harvested in luciferase lysis buffer. The luciferase and β-Gal activities were determined. TAZ exerts 6-fold increase in the transcriptional activity of Runx1 and 60-fold increase when MOZ and TAZ are present.

(B) The co-activator activities of TAZ and MOZ on Runx1 in ROS17.2/8 cells. GM-CSF-Luc plasmid was co-transfected with β-Gal, an internal control plasmid. Luciferase assays were performed as described in panel A. Both MOZ and TAZ mediate 2-fold increase in Runx1-dependent transcription.

(C) The co-activator activity of TAZ is PPXY motif-dependent, while the activity of MOZ is not. Y358A (PPPY to PPPA) mutant of Runx1 was used for transfection into ROS17.2/8 cells. Luciferase assays were performed parallel to those in panel B. MOZ can still activate mutant Runx1-dependent transcription while TAZ losses its activity.

(D) The synergy between TAZ and MOZ is dependent on the PPXY motif of Runx1. Luciferase assay were performed as described in as panel A. TAZ alone increases the activity of mutant Runx1 for 2-fold. The presence of MOZ, together with TAZ, causes 2-fold increase in the activity of mutant Runx1 (comparing 60-fold when using wild type Runx1).

Figure 2

Runx-dependent transcription is responsive to PKC signaling pathway

The 6OSE2-Luc reporter was co-transfected with plasmid for Runx2 protein. β-Gal plasmid was also transfected as an internal control. 24 h after transfection, PMA was added into the culture medium at a concentration of 50 ng/ml. PKC inhibitors GÖ6983 and GÖ6976 were added along with PMA. An additional 24 h after adding the chemicals, cells were harvested in luciferase lysis buffer. Luciferase and β-gal activities were determined. PMA increases the transcriptional activity of Runx2, this increase is blocked by PKC inhibitors GÖ6983 and GÖ6976.

Figure 3

Runx-dependent transcription is responsive to MAPK kinase signaling pathway Plasmids for 6OSE2-Luc reporter and Runx2 protein were transfected into ROS17/2.8 cells with or without MEK1dd, a constitutively active form of MEK1. β-Gal was also transfected as an internal control. 24 h after transfection, cells were harvested in luciferase lysis buffer. Luciferase and β-gal activities were determined. Constitutively active MEK1 upregulates the transcriptional activity of Runx2 by 6- to 9-fold in ROS17/2.8 cells. MEK1dd did not modify the protein expression levels of co-transfected plasmids.

Figure 4

PMA and MEK1dd stimulate the co-factor complex formation

In lanes 1 to 3, Flag-tagged MOZ SM (the serine and methionine-rich domain of MOZ) and HA-tagged TAZ were co-transfected with (lane 2) or without (lanes 1 and 3) HA-tagged MEK1dd. In lane 4, only HA-TAZ was transfected as a negative control. 24 h after transfection, PMA (50 ng/ml) was added into the medium (lane 3). After an additional 24 h, cells were harvested in buffer K150 (20 mM sodium phosphate, pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors) followed by immunoprecipitation on M2 agarose. After washing 4 times in Buffer K150, proteins were eluted by Flag peptide. Eluted proteins were resolved in SDS-PAGE followed by Western blotting. TAZ interacts with MOZ SM. Both PMA and MEK1dd enhance the interaction between MOZ SM and TAZ. The different protein levels between each transfection were due to variation of amount of plasmids, which did not alter the conclusion.

Figure 5

PMA upregulates Runx2-mediated endogenous GM-CSF level

HEK293 cells were transfected with the expression plasmids for indicated proteins. β-Gal plasmid was transfected as an internal control. 24 h after transfection, cells were treated with PMA (50 ng/ml) as indicated. After an additional 24 h, cells were harvested and ELISA was performed according to the manufacture's instructions. Without PMA, Runx2 increases CM-CSF levels for 2- to 3- fold, and TAZ mediates an additional 30% increase on the transcriptional activity of Runx2. However, 24 h of PMA treatment induces a 10-fold increase of GM-CSF levels in the presence of Runx2, and co-transfection of TAZ causes an additional increase. No effect of MOZ is observed before or after PMA treatment.

Figure 6

Model illustrating that MOZ and TAZ work synergistically and that the synergy is responsive to PMA and MAPK signaling

(A) TAZ and MOZ are known co-activators for Runx proteins. Without stimulation by signaling pathways, the transcriptional activity of Runx2 is low. TAZ, MOZ and Runx make loose interactions.

(B) In response to PKC and MAPK signaling, TAZ, MOZ and Runx2 form a tighter protein complex, resulting in a stronger transcription of target genes (indicated by larger arrow).

Supplementary figure

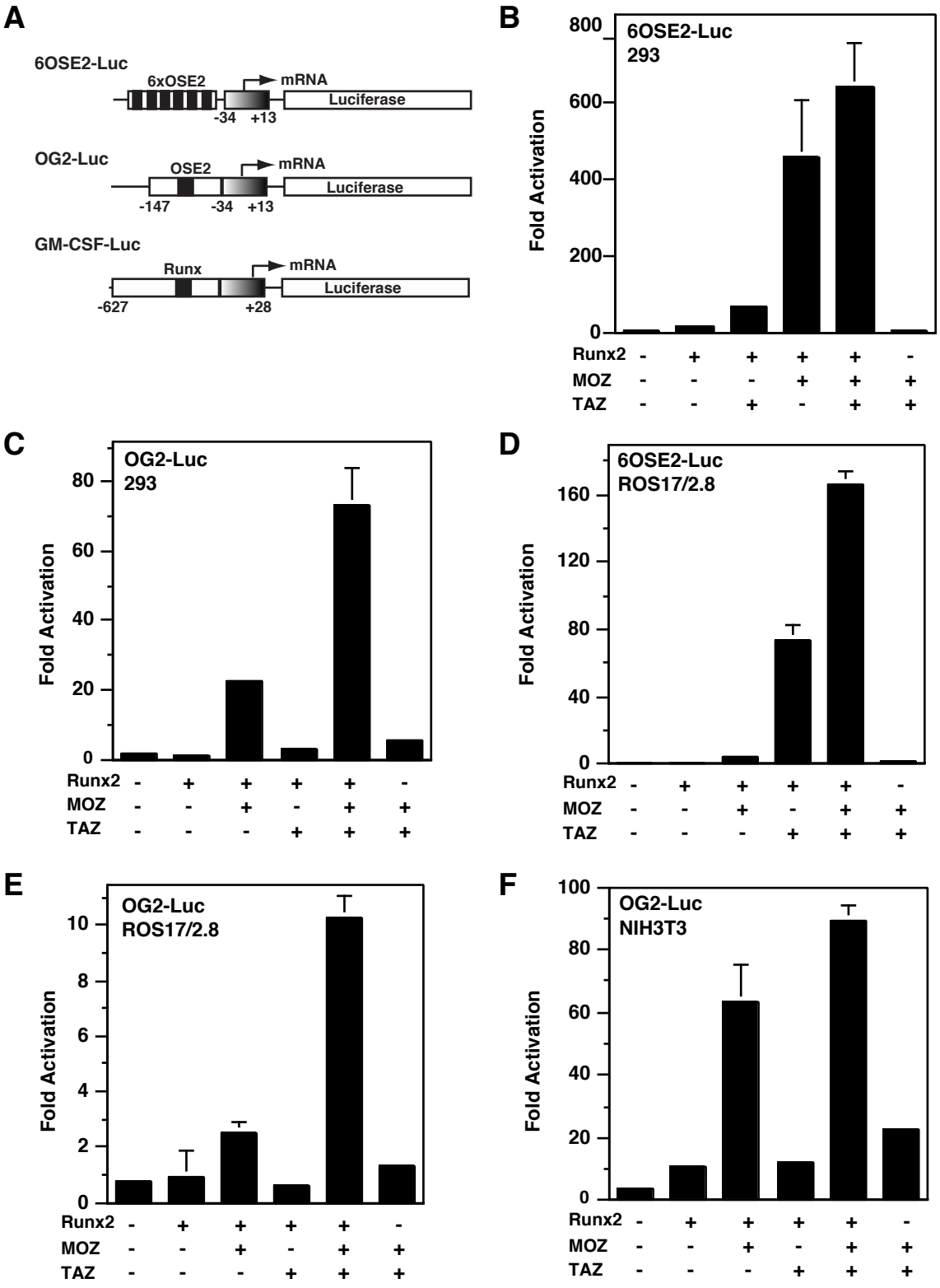
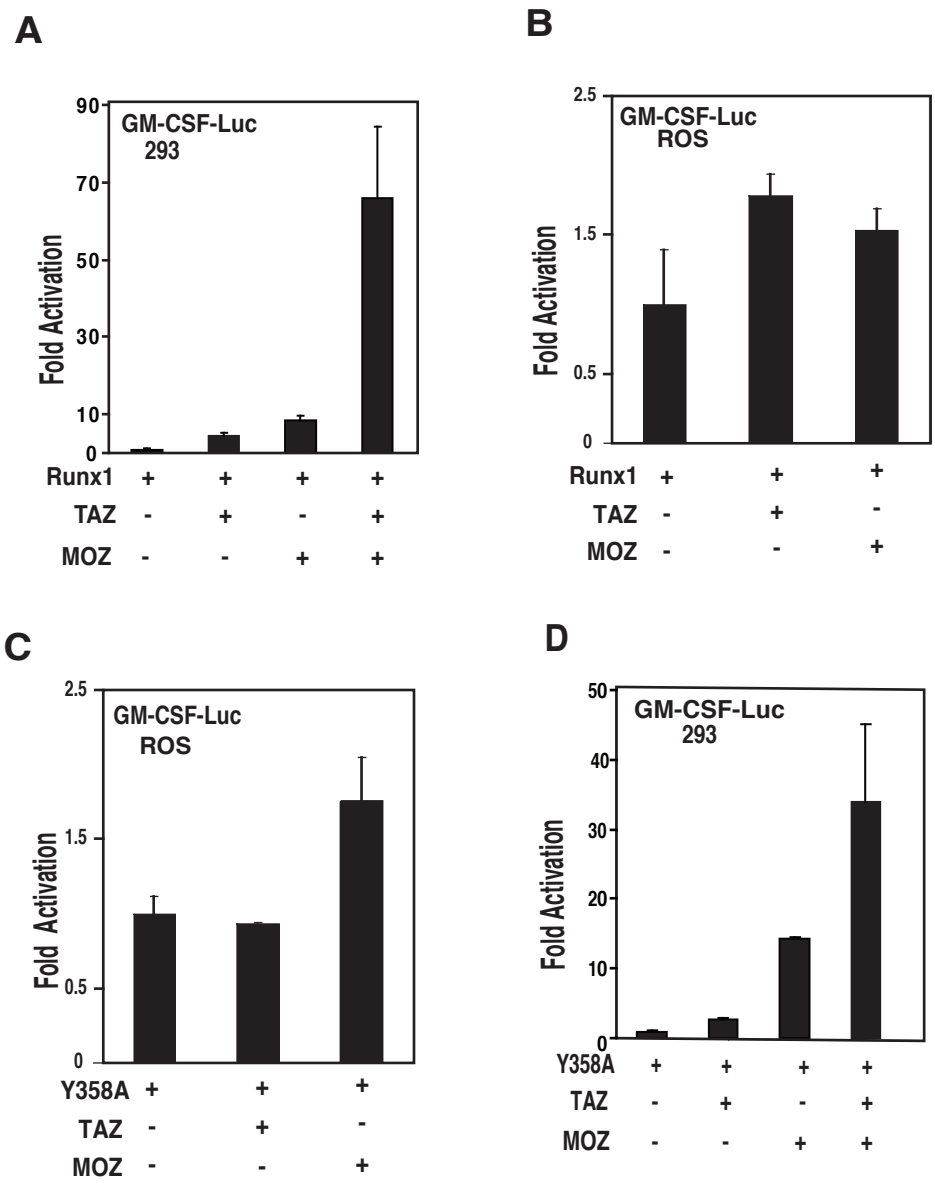


Figure 1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GM-CSF-Luc
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Fold Activation
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50
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B
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Fold Activation
2.5
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Runx1 + + +
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Figure 2

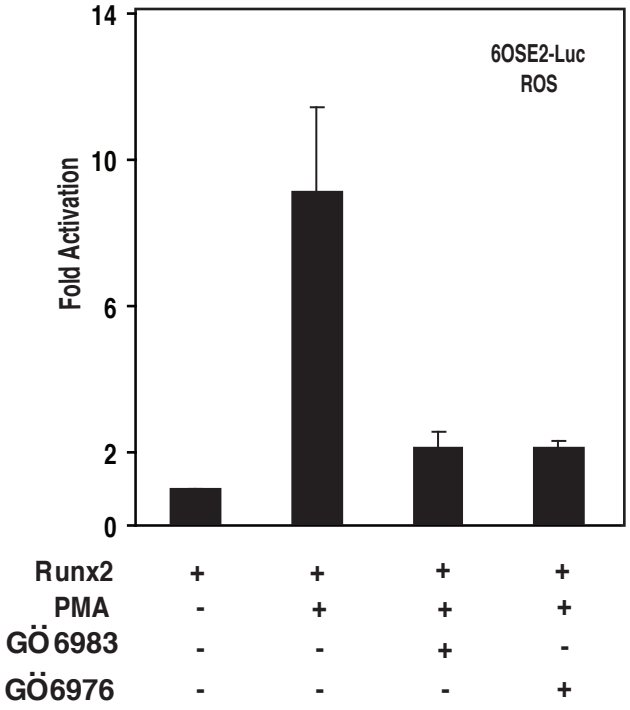
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Figure 3

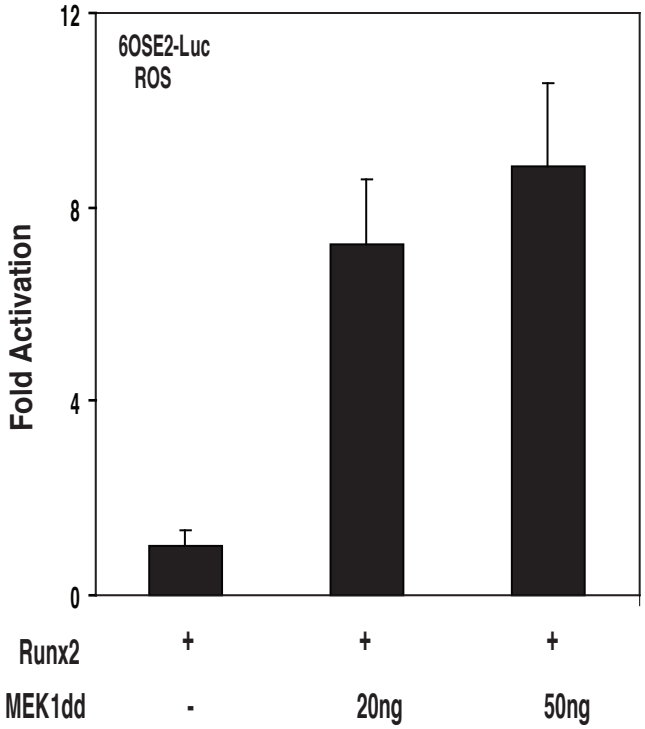
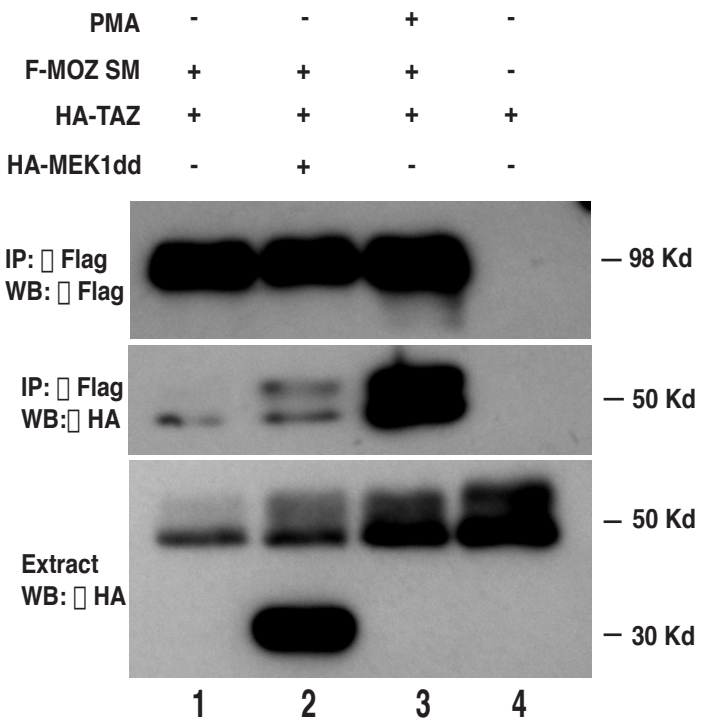


Figure 4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HA-TAZ + + + +
HA-MEK1dd - + - -
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IP: Flag
WB: HA
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Extract
WB: HA
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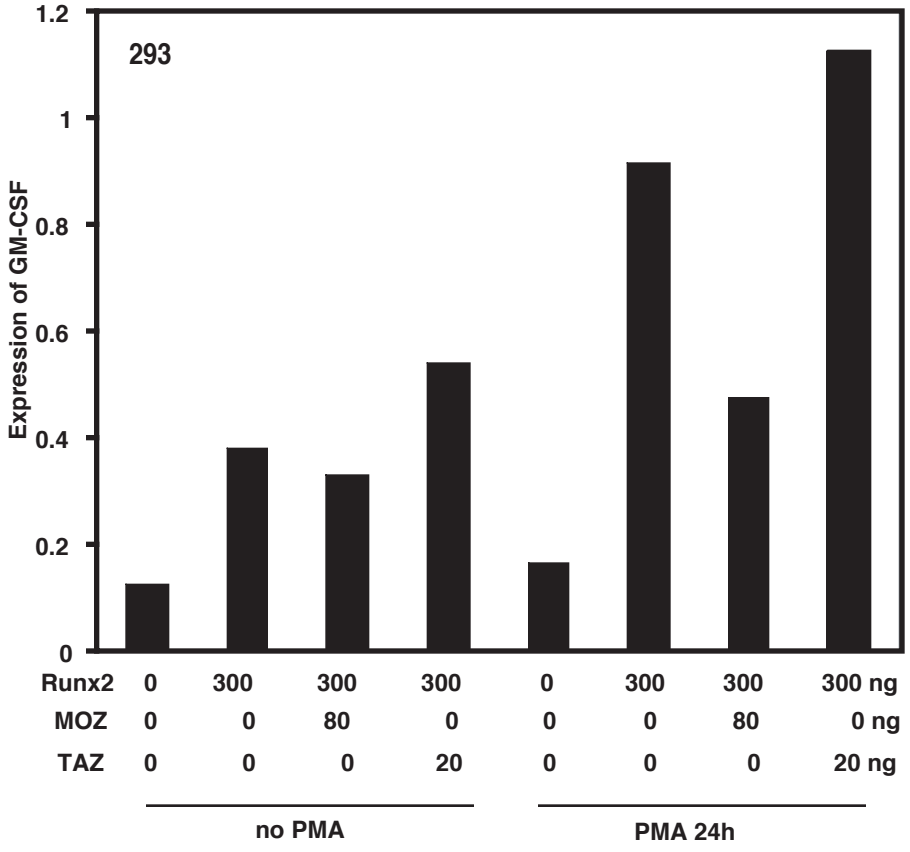
Figure 5


293
Expression of GM-CSF
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1
0.8
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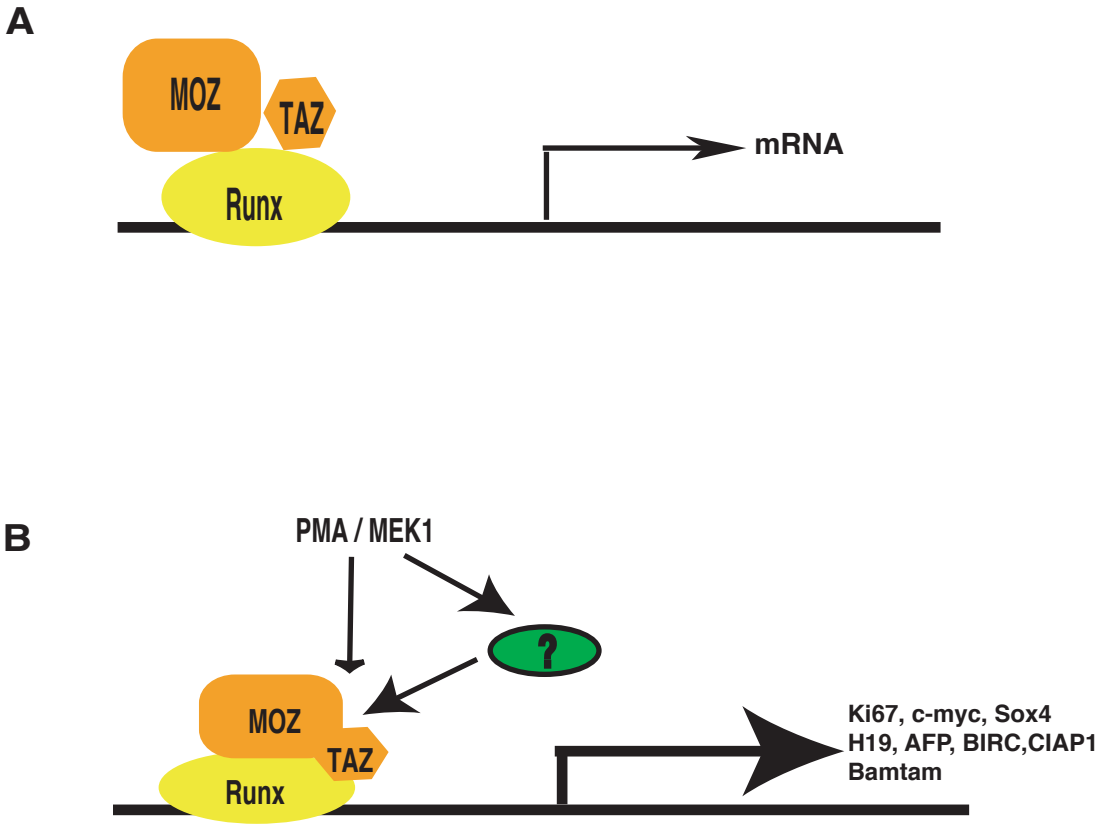
MOZ 0 0 80 0 0 0 80 0 ng
TAZ 0 0 0 20 0 0 0 20 ng
no PMA
PMA 24h

Figure 6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Ki67, c-myc, Sox4
H19, AFP, BIRC,CIAP1
Bamtam

Chapter 3

Different histone deacetylases target Runx transcription factors through their co-activators

Minghong Xu and Xiang-Jiao Yang

Preface

In Chapter 2, I showed evidence suggesting that MOZ and TAZ are recruited by the Runx proteins to form a co-activator complex, and this complex is stabilized by PKC and MAPK signaling. The ELISA experiments detecting endogenous GM-CSF provided further evidence that PKC signaling is stimulatory for co-activator activity of TAZ. These observations further support that TAZ is a signal responsive co-activator. However, the dual role of TAZ and YAP in transcriptional regulation necessitates studies to identify co-factors with transcriptional repressive properties. In light of the findings that TAZ is a co-activator for Runx2 and that HDAC4 is a key repressor for Runx2, we reasoned that HDACs might target Runx2 through multiple mechanisms, e.g. via co-activators like TAZ and YAP. This chapter focuses on whether and how HDACs are recruited by TAZ.

Abstract

Runx family transcription factors are a group of Runt domain-containing proteins that are important in many key biological processes and cancer development. Understanding the regulation of these proteins is of great importance for many key biological processes such as hematopoiesis, osteogenesis, etc. HDAC4 knockout mice exibit severe skeletal deformities, and HDAC4 binds to Runx2 and represses its transcriptional activity, suggesting that Runx2 is the *in vivo* target of HDACs. We extended this line of research by analyzing all classical HDACs and all Runx proteins. The results showed that both class I and II HDACs regulated Runx-dependent transcription. Furthermore, a new pathway in which Runx family proteins were regulated by HDACs is defined --- through their co-factors. We found that TAZ, YAP and CBP, three co-factors of Runx proteins, are the targets of HDACs. Lastly, we provided a mechanistic insight that acetylation and deacetylation of TAZ affects the function of TAZ.

Introduction

Runx proteins are transcription factors containing the Runt DNA binding domain. The family consists of Runx1, Runx2 and Runx3. Runx1 is important in hematopoiesis and sensory neuron development (Chen et al., 2006a; Chen et al., 2006b; Inoue et al., 2003; Kramer et al., 2006), Runx2 is essential for bone morphogenesis (Ducy, 2000; Ducy et al., 1999; Ducy et al., 1997; Harada et al., 1999; Karsenty et al., 1999) and Runx3 plays important roles in the immune system and gastric epithelium transformation, although this has been disputed (Brenner et al., 2004; Li et al., 2004; Li et al., 2002). Besides these important physiological functions, both gain-of-function and loss-of-function mutations of Runx genes result in various types of cancer. For example, Runx1-ETO was found to be causative in 10-20% of acute myeloid leukemia cases, the TEL-Runx1 fusion gene was found in 20-25% of childhood lymphoid leukemia and all three Runx members are the frequent targets of retrovirus insertion in retrovirus-induced leukemia models (Blyth et al., 2005).

Like many other transcription factors, Runx proteins possess a DNA-binding domain as well as activation and repression domains. So far, many molecules have been identified as co-activators, such as Rb proteins (Thomas et al., 2001), MOZ, MORF (Kitabayashi et al., 2001; Pelletier et al., 2002) and TAZ (Cui et al., 2003). Not only are Runx proteins causative to cancer and many diseases, but also their co-factors have also been closely linked. Among the co-activators, TAZ was discovered for its ability to bind to 14-3-3. Through its WW domain, TAZ binds to the PPXY motif of target transcription factors and supposedly contacts the basal transcription machinery via the C-terminal

activation domain (Kanai et al., 2000). Besides Runx proteins, several other transcription factors, including TBX5, PAX5 and TEF (Mahoney et al., 2005; Murakami et al., 2005; Murakami et al., 2006), have also been shown to utilize TAZ as a co-activator, indicating that TAZ is important in organ development and disease.

Many co-repressors of Runx proteins have also been identified, including Grouch/TLE (transducin-like enhancer of split) (Javed et al., 2000; Yarmus et al., 2006) , mSin3a (Imai et al., 2004; Lutterbach et al., 2000), YAP (Zaidi et al., 2004), and histone deacetylases (HDACs) (Vega et al., 2004). HDACs are enzymes that catalyze the removal of acetyl moieties from many proteins including histones and non-histone proteins. Regarding bone development, clear evidence comes from HDAC4 knockout mice in which the skeletal system is severely affected (Vega et al., 2004). Biochemical experiments show that HDAC4 binds to Runx2 and represses its transcriptional activity. This finding shows that the repressors exert their roles in disease and development by inhibiting the transcriptional activities of transcription factors. An unaddressed question is whether this repression acts through co-activators as well.

Considering the fact that both HDAC4 and 5 contain the PPSY motif, we strongly favor the scenario that the modulation of Runx-dependent transcription by HDAC4 and 5 is through their modulating the co-activator TAZ as well. Hence, we performed biochemical analysis to investigate such possibilities and showed that both class I and II HDACs modulated Runx-dependent transcription through their co-activators including TAZ.

Materials and Methods

Mutagenesis of HDAC5 — HDAC5 Y229F mutant was generated by site-directed mutagenesis as described previously.

Immunofluorescence microscopy — Tagged expression plasmids were transfected into HEK293 cells using Superfect (Qiagen). 48 h after transfection, cells were fixed with 0.2% paraformaldehyde followed by incubation with anti-HA antibody at room temperature for 20 min. After being washed 6 times with PBS (pH 7.2), cells were stained with Cy3-conjugated anti-rabbit immunoglobulin G antibody (Alex555, Invitrogen). Cells were also stained with Hoechst 33528 to visualize the nuclei. Expression of GFP fusion proteins was determined by green fluorescence microscopy.

*Protein-protein interaction and Western blot*_For protein expression in HEK293 cells, 10 μg of expression plasmids was used to transfect 10^6 cells (in a 10-cm diameter dish) with 24 μl of SuperFect transfection reagent (Qiagen). After 48 h, cells were washed twice with PBS and collected in 1 ml of buffer B (20 mM Tris-HCl [pH 8.0], 10% glycerol, 5 mM $MgCl_2$, 0.1% NP-40, protease inhibitors) containing 0.15 M KCl or Buffer K (20 mM sodium phosphate, pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors). The same buffer was used for washing M2 agarose beads. The bound proteins were eluted in the same buffer. Eluted proteins were resolved in 8% to 10% SDS-PAGE. Western blotting was performed on the nitrocellulose membrane containing transferred proteins from the gel. 1:2000 dilution for anti-HA or anti-Flag (Sigma) antibody was used. Briefly, antibodies were incubated in PBST (PBS containing 0.015% Tween 20) with 20% horse serum for 2 h at room temperature followed by washing for 1 h with

PBST. The secondary antibody with a dilution of 1:1000 was incubated with the membrane for 1 h at room temperature followed by washing for 1 h. For anti-acetyl lysine antibody (Cell Signaling), 1:400 dilution was used with the rest of the procedures being the same except that the incubation with the first antibody was performed at 4°C for a overnight period.

Reporter gene assays — SuperFect transfection reagent (Qiagen) was used to transiently transfect a luciferase reporter plasmid (50 to 200 ng) and/or mammalian expression plasmids (50 to 200 ng) into HEK293 or ROS17/2.8 cells. pBluescript KSII(+) was used to normalize the total amount of plasmids used in each transfection and β-Gal expression plasmid (50 ng) was co-transfected as an internal control. After 24 to 48 h, cells were lysed and luciferase reporter activity was determined using D-(L)-luciferin (Boehringer Mannheim) as the substrate. β-Galactosidase activity was measured with Galacto-Light Plus (Tropix) as the substrate. The chemiluminescence from activated luciferin or Galacto-Light Plus was measured on a Luminometer plate reader (Dynex). Each transfection was performed at least three times.

Results

Class II HDACs interact with TAZ

Sequence inspection reveals that HDAC5 has a PPSY motif, a potential site for binding to WW domain-containing proteins such as TAZ and YAP. We investigated if HDAC5 could interact with TAZ and YAPΔ, a truncation mutant of YAP lacking the proline-rich domain. To test this, we performed co-immunoprecipitation experiments by overexpressing these proteins in HEK293 cells. The results showed that HDAC5 bound to TAZ as well as YAPΔ (Fig. 1A). We next analyzed the importance of the PPSY motif for the binding. To address this, the PPSY motif of HDAC5 was mutated into PPSF, and this mutant was used in co-immunoprecipitation together with the wild type HDAC5 (Fig. 1B). The mutant HDAC5 could still bind to TAZ, indicating that there is more than one motif involved in the interaction.

HDAC5 belongs to class II HDACs. All members of this class possess an extended N-terminal region. Sequence comparison within this region reveals high homology among members. This similarity suggests similar biochemical properties. So we tested other members of class II HDACs in binding to TAZ. As expected, we found that HDAC7 also bound to TAZ, and was a stronger binding partner for TAZ compared to HDAC4 and 5 (Fig. 1C). Although we could not detect the interaction between HDAC4 and TAZ, the immunofluorescence experiments showed that these two proteins colocalized in both the nucleus and the cytoplasm (Fig. 2A). These facts indicate that the affinity between HDAC4 and TAZ might be regulated and/or prone to condition changes. Also, as shown in Fig. 2, HDAC5 colocalized with TAZ in the nucleus (Fig. 2B). Taken

together, our results demonstrate that class II HDACs are the interaction partners for TAZ and YAPΔ.

Class II HDACs represses TAZ mediated co-activation

To analyze the functional consequences of the association between TAZ and class II HDACs, we performed reporter gene assay using 6OSE2-Luc and GM-CSF-Luc reporters. When these reporters were co-transfected with Runx proteins into HEK293 cells, Runx proteins caused a 3- to 5- fold transcriptional induction on those reporters. When TAZ was also transfected, an additional 5- to 10- fold activation was observed (Fig. 3A, 3B). As expected, a strong repression was observed when HDAC4 and 5 were co-expressed (Fig. 3). We repeated the same experiments in ROS17/2.8 cells. The results were similar (Fig. 3C). These data demonstrate that class II HDACs are co-repressors for TAZ-mediated co-activation.

The effects of class II HDACs on the TAZ-dependent co-activation in our assays can be attributable to the possibility that HDACs act directly on Runx protein themselves. To show the direct roles of class II HDACs on TAZ, we fused TAZ to the DNA-binding domain of Gal4 (Gal4-DBD). As shown in Fig. 4E, both HDAC4 and 5 had minimal effects on the basal promoter activity of Gal4-TK-Luc, but caused a 3- to 5- fold decrease on the activity of Gal4-TAZ. This result showed that class II HDACs directly repressed TAZ-dependent co-activation.

Class II HDACs regulate the transcriptional activities of Runx family proteins

The study on the HDAC4 knockout mouse model shows that HDAC4 controls chondrocyte hypertrophy by regulating Runx2-dependent transcription (Vega et al., 2004). We wanted to investigate whether other members of class II HDACs could act on Runx in a similar fashion. Our detailed analysis showed that HDAC 5 and 7 also repressed Runx-dependent transcription potently (Fig. 4D, 4E).

Since the Runt domain is the site of Runx2 for binding to HDAC4, thereby mediating the inhibitory effect (data not shown), and the structure of Runx family proteins is highly homologous, especially in the runt domain, we asked if other Runx proteins could bind to HDAC4 and other members of class II HDACs such as HDAC5 and 7. To test this, we performed co-immunoprecipitation by overexpressing Runx1 and Runx3 with class II HDACs. The results showed that both Runx1 and Runx3 bound to HDAC4, 5 and 7 (Fig. 4).

HDAC3 targets co-activators of Runx

The previous findings prompted us to consider the possibility that class I HDACs could also modulate the co-activator activity of TAZ. To test this, we performed the co-immunoprecipitation by expressing HDAC3 and TAZ in HEK293 cells. The result showed strong physical interaction between these two proteins (Fig. 5A). Immunofluorescence microscopy showed co-localization of these two proteins both in the nucleus and the cytoplasm (data not shown). Furthermore, we investigated if HDAC3 could target other co-activators of Runx proteins. We tested known co-activators MOZ and CBP. The co-immunoprecipitation experiments showed strong binding between MOZ and HDAC3 when co-expressed in HEK293 cells (Fig. 5A). In the reporter gene

assay, however, we did not observe the repression by HDAC3 on MOZ-dependent co-activation (data not shown). We also performed reporter gene assays to investigate if HDAC3 could repress CBP-dependent co-activation. As shown in Fig. 5B, CBP activated Runx2-dependent transcription for 2-fold. But when HDAC3 was present, the activation was inhibited. These preliminary findings support that class I HDACs such as HDAC3 also targets co-activators of Runx.

Acetylation plays a role in TAZ-dependent co-activator activity

The physical interaction between TAZ and HDACs led us to consider if TAZ could be the substrate of HDACs. For this, we first investigated if TAZ was acetylated by histone acetylases both *in vitro* and *in vivo*. We tested MOZ, MORF and p300 *in vitro*. As shown in Fig. 6A, TAZ was specifically acetylated by p300. Next, we investigated whether CBP or p300 could acetylate TAZ *in vivo*. We co-transfected F-TAZ and HA-CBP into HEK293 cells. 48 h later, TAZ was purified in the presence of both nicotinamide (NAD, class III HDAC inhibitor) and sodium butyrate (Bu, class I and II HDAC inhibitor) to inhibit different classes of HDACs. The eluted proteins were subjected to Western blotting with either anti-acetyl lysine or anti-Flag antibody. The results showed that TAZ and YAPΔ were efficiently acetylated by CBP *in vivo* (Fig. 6B, 6C). We did not detect the physical binding between CBP and TAZ in the co-immunoprecipitation studies. But in the immunofluorescence experiment, we found that TAZ colocalized well with CBP in the nuclear dots in HEK293 cells (Fig. 6F). This suggests that CBP resides in the same nuclear domains with and acetylates TAZ.

We also sought to identify the responsible deacetylases. To achieve this, we performed Western blot study using Flag-tagged TAZ purified under conditions wherein different deacetylase inhibitors were added (Fig. 6D). The result showed that acetylated TAZ was detected in samples treated with either NAD or sodium butyrate, suggesting that TAZ is the target of multiple classes of HDACs.

So far, we have shown that TAZ is the substrate for both CBP and HDACs. Protein acetylation plays profound roles in the regulation of protein functions. We wanted to know if acetylation played a role in regulating the co-activator activity of TAZ. The reporter gene assay showed that there was synergy between TAZ and CBP on Runx-dependent transcription (Fig. 6E), suggesting a possible link between TAZ acetylation and enhanced co-activator activity.

Discussion

Class II HDACs regulate transcriptional activities of Runx proteins through their co-activator TAZ

HDAC4 knockout and transgene mice exhibit severe skeletal defects (Vega et al., 2004). It turned out that deregulation of Runx2 function is responsible for this phenotype. It was found that HDAC4 binds to the Runt domain of Runx2 and abolishes its DNA binding activity, thereby repressing the transcriptional activity of Runx2 (Vega et al., 2004). Our study added that the repression on Runx-dependent transcription by HDAC4 could also be due to its action on co-activators. Our data demonstrate that not only HDAC4, but also other members of the HDAC family target TAZ, directly repressing its activation as shown by the reporter gene assay experiments on Gal4-TK-Luc promoter (Fig. 3D). Taken together, our data demonstrate the direct role of class II HDACs in regulating transcriptional activities of Runx proteins mediated by TAZ.

As mentioned earlier, TAZ is also a co-activator of other proteins such as TBX5 and PAX3 (Murakami et al., 2005; Murakami et al., 2006). But the HDAC4 knockout model did not show overt abnormalities in those systems where TBX5 and PAX3 are expressed. The redundancy of HDACs might be the culprit. It would be an interesting study to perform in situ hybridization to localize HDACs in the development of the embryo as well as spatial distributions at certain stages. This will help to point out the true physiological partners, and shed light on the uncovered functions of HDACs.

Class I HDACs regulates co-factors of Runx

Some evidence suggested the physical and functional association between class I and II HDACs (Fischle et al., 2002). We also found that there are at least three regions in HDAC4 that interact with HDAC3 (data not shown). This indicates that different HDACs collaborate and form a large repressor complex to achieve repression. Our data showed that HDAC3, a member of class I HDAC, could bind to TAZ and YAP. Furthermore, we showed that other co-activators of Runx such as CBP/p300 were under control of HDAC3. These findings strongly suggest that different HDACs collaborate to regulate Runx-dependent transcription through co-activators. CBP and p300 are proteins with intrinsic histone acetyltransferase activities and undergo self-acetylation. Self-acetylation is essential for their activation and HAT activities (Thompson et al., 2004). As HDAC3 can be the deacetylase for some non-histone proteins, we hypothesize that HDAC3 inactivates CBP/p300 by deacetylation.

Role of acetylation and deacetylation in functional regulation of TAZ

Histone deacetylases were first identified for their abilities to remove acetyl groups from histones (Taunton et al., 1996), and later the substrates were expanded to include non-histone proteins (Yang, 2004). In our assay systems, DNA is transiently transfected plasmid with minimal histone coating. So we tend to think histone modification in the repression of reporter gene activation is less important. The study on HDAC4 knockout mice provided one important mechanism by which HDACs impede the DNA binding of Runx (Vega et al., 2004), which is consistent with what we have observed. But regarding TAZ, a different scenario should apply especially considering that HDAC4 and 5 repress TAZ-mediated co-activation on Gal4-TK-Luc reporter. In this case, we favor the notion

that direct acetylation and deacetylation of TAZ play a role. As shown in Fig. 4, the clear nuclear co-localization of these two proteins, and more importantly, the synergy between CBP and TAZ, suggest that CBP-mediated TAZ acetylation plays a role in regulating its activity. In the future, mapping of the acetylation site of TAZ and determination of the transcriptional activities of those mutants will further clarify the role of acetylation and deacetylation in functional regulation of TAZ.

TAZ forms a co-repressor complex with other co-repressors

TAZ is known to be a co-activator. But the property that TAZ associates with HDACs provides clues that TAZ could recruit HDACs to become a repressor. Indeed, TAZ represses PPARγ-dependent transcription, which is important in the differentiation of mesenchyma into fatty tissue (Hong and Yaffe, 2006). Recently, another group reported that ligand-dependent sumoylation of PPARγ recruits HDAC3, thus targeting PPARγ to the N-CoR repressor complex (Pascual et al., 2005). Our finding that TAZ interacts with HDAC3 indicates that TAZ, HDAC3 and PPARγ form a protein complex in response to cellular stimuli.

In conclusion, our biochemical studies provided molecular basis for TAZ to behave as a repressor. In the future, the signaling pathways that control the function of TAZ should be identified, particularly those could possibly mediate the conversion of TAZ between a co-activator and a repressor.

References

Blyth, K., Cameron, E. R., and Neil, J. C. (2005). The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer *5*, 376-387.

Brenner, O., Levanon, D., Negreanu, V., Golubkov, O., Fainaru, O., Woolf, E., and Groner, Y. (2004). Loss of Runx3 function in leukocytes is associated with spontaneously developed colitis and gastric mucosal hyperplasia. Proc Natl Acad Sci U S A *101*, 16016-16021.

Chen, A. I., de Nooij, J. C., and Jessell, T. M. (2006a). Graded activity of transcription factor Runx3 specifies the laminar termination pattern of sensory axons in the developing spinal cord. Neuron *49*, 395-408.

Chen, C. L., Broom, D. C., Liu, Y., de Nooij, J. C., Li, Z., Cen, C., Samad, O. A., Jessell, T. M., Woolf, C. J., and Ma, Q. (2006b). Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron *49*, 365-377.

Cui, C. B., Cooper, L. F., Yang, X., Karsenty, G., and Aukhil, I. (2003). Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. Mol Cell Biol *23*, 1004-1013.

Ducy, P. (2000). Cbfa1: a molecular switch in osteoblast biology. Dev Dyn *219*, 461-471.

Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M., and Karsenty, G. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev *13*, 1025-1036.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell *89*, 747-754.

Fischle, W., Dequiedt, F., Hendzel, M. J., Guenther, M. G., Lazar, M. A., Voelter, W., and Verdin, E. (2002). Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell *9*, 45-57.

Harada, H., Tagashira, S., Fujiwara, M., Ogawa, S., Katsumata, T., Yamaguchi, A., Komori, T., and Nakatsuka, M. (1999). Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem *274*, 6972-6978.

Hong, J. H., and Yaffe, M. B. (2006). TAZ: a beta-catenin-like molecule that regulates mesenchymal stem cell differentiation. Cell Cycle *5*, 176-179.

Imai, Y., Kurokawa, M., Yamaguchi, Y., Izutsu, K., Nitta, E., Mitani, K., Satake, M., Noda, T., Ito, Y., and Hirai, H. (2004). The corepressor mSin3A regulates phosphorylation-induced activation, intranuclear location, and stability of AML1. Mol Cell Biol *24*, 1033-1043.

Inoue, K., Ozaki, S., Ito, K., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S. C., Yamashita, N., Itohara, S., Kudo, N., and Ito, Y. (2003). Runx3 is essential for the target-specific axon pathfinding of trkc-expressing dorsal root ganglion neurons. Blood Cells Mol Dis *30*, 157-160.

Javed, A., Guo, B., Hiebert, S., Choi, J. Y., Green, J., Zhao, S. C., Osborne, M. A., Stifani, S., Stein, J. L., Lian, J. B., *et al.* (2000). Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. J Cell Sci *113 (Pt 12)*, 2221-2231.

Kanai, F., Marignani, P. A., Sarbassova, D., Yagi, R., Hall, R. A., Donowitz, M., Hisaminato, A., Fujiwara, T., Ito, Y., Cantley, L. C., and Yaffe, M. B. (2000). TAZ: a

novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. Embo J *19*, 6778-6791.

Karsenty, G., Ducy, P., Starbuck, M., Priemel, M., Shen, J., Geoffroy, V., and Amling, M. (1999). Cbfa1 as a regulator of osteoblast differentiation and function. Bone *25*, 107-108.

Kitabayashi, I., Aikawa, Y., Nguyen, L. A., Yokoyama, A., and Ohki, M. (2001). Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. Embo J *20*, 7184-7196.

Kramer, I., Sigrist, M., de Nooij, J. C., Taniuchi, I., Jessell, T. M., and Arber, S. (2006). A role for Runx transcription factor signaling in dorsal root ganglion sensory neuron diversification. Neuron *49*, 379-393.

Li, J., Kleeff, J., Guweidhi, A., Esposito, I., Berberat, P. O., Giese, T., Buchler, M. W., and Friess, H. (2004). RUNX3 expression in primary and metastatic pancreatic cancer. J Clin Pathol *57*, 294-299.

Li, Q. L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X. Z., Lee, K. Y., Nomura, S., Lee, C. W., Han, S. B., *et al*. (2002). Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell *109*, 113-124.

Lutterbach, B., Westendorf, J. J., Linggi, B., Isaac, S., Seto, E., and Hiebert, S. W. (2000). A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. J Biol Chem *275*, 651-656.

Mahoney, W. M., Jr., Hong, J. H., Yaffe, M. B., and Farrance, I. K. (2005). The transcriptional co-activator TAZ interacts differentially with transcriptional enhancer factor-1 (TEF-1) family members. Biochem J *388*, 217-225.

Murakami, M., Nakagawa, M., Olson, E. N., and Nakagawa, O. (2005). A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. Proc Natl Acad Sci U S A *102*, 18034-18039.

Murakami, M., Tominaga, J., Makita, R., Uchijima, Y., Kurihara, Y., Nakagawa, O., Asano, T., and Kurihara, H. (2006). Transcriptional activity of Pax3 is co-activated by TAZ. Biochem Biophys Res Commun *339*, 533-539.

Pascual, G., Fong, A. L., Ogawa, S., Gamliel, A., Li, A. C., Perissi, V., Rose, D. W., Willson, T. M., Rosenfeld, M. G., and Glass, C. K. (2005). A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. Nature *437*, 759-763.

Pelletier, N., Champagne, N., Stifani, S., and Yang, X. J. (2002). MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. Oncogene *21*, 2729-2740.

Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408-411.

Thomas, D. M., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell *8*, 303-316.

Thompson, P. R., Wang, D., Wang, L., Fulco, M., Pediconi, N., Zhang, D., An, W., Ge, Q., Roeder, R. G., Wong, J., *et al.* (2004). Regulation of the p300 HAT domain via a novel activation loop. Nat Struct Mol Biol *11*, 308-315.

Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J. M., Richardson, J. A., *et al*. (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell *119*, 555-566.

Yang, X. J. (2004). Lysine acetylation and the bromodomain: a new partnership for signaling. Bioessays *26*, 1076-1087.

Yarmus, M., Woolf, E., Bernstein, Y., Fainaru, O., Negreanu, V., Levanon, D., and Groner, Y. (2006). Groucho/transducin-like Enhancer-of-split (TLE)-dependent and -independent transcriptional regulation by Runx3. Proc Natl Acad Sci U S A *103*, 7384-7389.

Zaidi, S. K., Sullivan, A. J., Medina, R., Ito, Y., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. (2004). Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. Embo J *23*, 790-799.

Figure 1

Class II HDACs interact with WW domain-containing proteins

(A) Binding of HDAC5 to WW domain-containing proteins. Expression plasmids for Flag-tagged TAZ, YAP truncation mutant (YAPΔ) and another WW domain-containing protein Smurf, an E3 ubiquitin ligase, were co-transfected with HA-HDAC5 into HEK293 cells. 48 h after transfection, cells were harvested in buffer K150 (20 mM sodium phosphate, pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors). Co-immunoprecipitation was performed by incubation of cell extracts with M2 agarose beads at 4°C for 2 h. After being washed 4 times using buffer K150, bound proteins were eluted by Flag peptide. Eluted proteins were resolved by SDS-PAGE, followed by Western blotting using either anti-HA or anti-Flag antibody. HDAC5 interacts with TAZ and YAPΔ. No interaction between Smurf and HDAC5 was detected.

(B) Multiple domains of HDAC5 bind to TAZ. The PPSY motif of HDAC5 was mutated to PPSF (Y229F). Plasmids for mutant and wild type HDAC5 proteins were co-transfected with Flag-TAZ into HEK293 cells, followed by co-immunoprecipitation and Western blot as described in panel A. Both wild type and mutant HDAC5 bind to TAZ.

(C) Class II HDACs interact with TAZ. Plasmids expressing Flag-tagged HDAC4, 5, 7 and MITR, a protein identical to the N-terminal portion of HDAC9, were co-transfected with HA-TAZ expression plasmid into HEK293 cells. Co-immunoprecipitation and Western blot were performed as described in panel A. Both HDAC5 and 7 bind to TAZ (lanes 3 and 4) while the affinity between HDAC4 and TAZ is not detectable in our buffer condition (lane 2).

Figure 2

Co-localization of HDAC4 and 5 with TAZ

(A) Expression plasmid for GFP-HDAC4 protein was co-transfected with HA-TAZ into HEK 293 cells. 48 h after transfection, cells were fixed by 0.2% paraformaldehyde. After being blocked in 1% BSA at room temperature for 15 min, cells were incubated with anti-HA antibody for 20 min at room temperature, followed by washing with PBS. Then, cells were incubated with Alex555 secondary antibody for 20 min at room temperature. Nucleus was stained with Hoechst. Images were processed using Adobe Photoshop 5. HDAC4 is cytoplasmic (Bottom panels) or forms nuclear dots (Upper panels). TAZ colocalizes with HDAC4 in both nucleus and cytoplasm.

(B) Expression plasmid for GFP-HDAC5 protein was co-transfected with HA-TAZ into HEK293 cells. 48 h later, immunoflurescence was performed as described in panel A. HDAC5 is nuclear in HEK293 cells. TAZ shows distribution in both nucleus and cytoplasm. Nuclear portion of TAZ colocalizes with HDAC5.

Figure 3

Class II HDACs repress TAZ-dependent co-activation

(A) 6OSE2-Luc was used as the reporter. Plasmids for indicated proteins were transfected into HEK293 cells. β-Gal plasmid was also transfected as an internal control. 48 h later, cells were lysed in luciferase buffer. Luciferase and β-Gal activities were determined. Both HDAC4 and 5 repress co-activator activity of TAZ.

(B) GM-CSF-Luc reporter was transfected into HEK293 cells along with other plasmids for indicated proteins. Luciferase assay was performed as described in panel A. Both HDAC4 and 5 repress co-activator activity of TAZ on GM-CSF-Luc reporter.

(C) 6OSE2-Luc reporter was transfected into ROS17/2.8. Plasmids for indicated proteins were also transfected along with β-Gal plasmid. Luciferase assay was performed as described in panel A. HDAC4, 5 and 7 inhibit the co-activator activity of TAZ.

(D) GAL4-TK-Luc was used as the reporter. Luciferase assay was performed as described in panel A. HDAC4 and 5 repress TAZ-dependent co-activation.

Figure 4

Class II HDACs bind to all members of the Runx family proteins and repress their transcriptional activities

(A) Binding of HDAC4 and 5 to Runx1 and 2. Expression plasmids for Flag-tagged Runx1 and Runx2 proteins were co-transfected with HA-HDAC4 or HDAC5 into HEK293 cells. 48 h after transfection, cells were harvested in buffer K150. Co-immunoprecipitation was performed as described previously. Runx1 interacts with both HDAC4 and 5. Runx2 binds to HDAC5 strongly, while the binding between HDAC4 and Runx2 is very weak.

(B) Class II HDACs interact with Runx1. Flag-tagged HDAC4, 5, 7 and MITR were transfected into HEK293 cells along with HA-Runx1. Co-immunoprecipitation and Western blot were performed as described in panel A of this figure. All three HDACs and MITR bind to Runx1.

(C) Class II HDACs interact with Runx3. Flag-tagged HDAC4, 5, 7 and MITR were transfected into HEK293 cells with HA-Runx3. Co-immunoprecipitation and Western blot were performed as described in panel A. Runx3 binds to all tested proteins.

(D) Luciferase assays show that class II HDACs repress Runx-dependent transcription. 6OSE2-Luc was used as reporter. Plasmids for indicated proteins were transfected into HEK293 cells. β-Gal was transfected as an internal control. 48 h later, cells were lysed in luciferase lysis buffer followed by determination of Luciferase and β-Gal activities. HDAC4, 5 and 7 repress Runx-dependent transcription on 6OSE2-Luc reporter.

(E) GM-CSF-Luc reporter was co-transfected into HEK293 cells along with plasmids for indicated proteins. β-Gal was transfected as an internal control. 48 h later, cells were

lysed in luciferase buffer followed by determination of Luciferase and β-Gal activities.

HDAC4, 5 and 7 inhibit Runx-dependent transcription on GM-CSF-Luc reporter.

Figure 5

HDAC3 targets co-factors of Runx proteins

(A) Interactions between HDAC3 with TAZ, MOZ and YAPΔ, a truncation mutant of YAP. The plasmids for indicated proteins were transfected into HEK293 cells. 48 h after transfection, cells were harvested in buffer K150 followed by co-immunoprecipitation and Western blot as described previously. HDAC3 shows strong binding with TAZ and MOZ. YAPΔ interacts with HDAC3 weakly.

(B) Luciferase assays show that HDAC3 targets CBP. The 6OSE2-Luc reporter was transfected into HEK293 cells along with the indicated plasmids. β-Gal plasmid was transfected as an internal control. Luciferase and β-Gal activities were determined 24 h after transfection as described previously. CBP activates Runx2-dependent transcription for 2- fold. This activation is repressed by HDAC3.

Figure 6

Acetylation and deacetylation regulate TAZ-mediated co-activation

(A) Specific acetylation of TAZ by p300. Flag-TAZ, expressed in and affinity-purified from insect cells, was used for in vitro acetylation by MORF, PCAF and p300 in the presence of [14-C]acetyl CoA. The HATs were also expressed in and affinity-purified from insect cells. Acetylated proteins were resolved by SDS-PAGE for subsequent autoradiography. Note the autoacetylation (marked "auto") of MORF, PCAF and p300. The acetylation sites for p300 were mapped to Lys 45 and 46 by mass spectrometry.

(B) TAZ is acetylated by CBP *in vivo*. Expression plasmid for Flag-tagged TAZ protein was co-transfected with that for HA-CBP into HEK293 cells. 24 h after transfection, sodium butyrate (Bu, class I and II HDAC inhibitor) and nicotinamide (NAD, class III HDAC inhibitor) were added into the culture medium as indicated. An additional 24 h after adding the HDAC inhibitors, cells were harvested in buffer K150 containing both inhibitors. Immunoprecipitation was performed by incubation of cell extracts with M2 agarose beads at 4°C for 2 h. After being washed 4 times using buffer K150 plus inhibitors, proteins were eluted by Flag peptide. Eluted proteins were resolved by SDS-PAGE, followed by Western blot using either anti-HA or anti-Flag antibody as well as anti-acetyl-lysine antibody. In the presence of Both HDAC inhibitors, CBP acetylates TAZ efficiently.

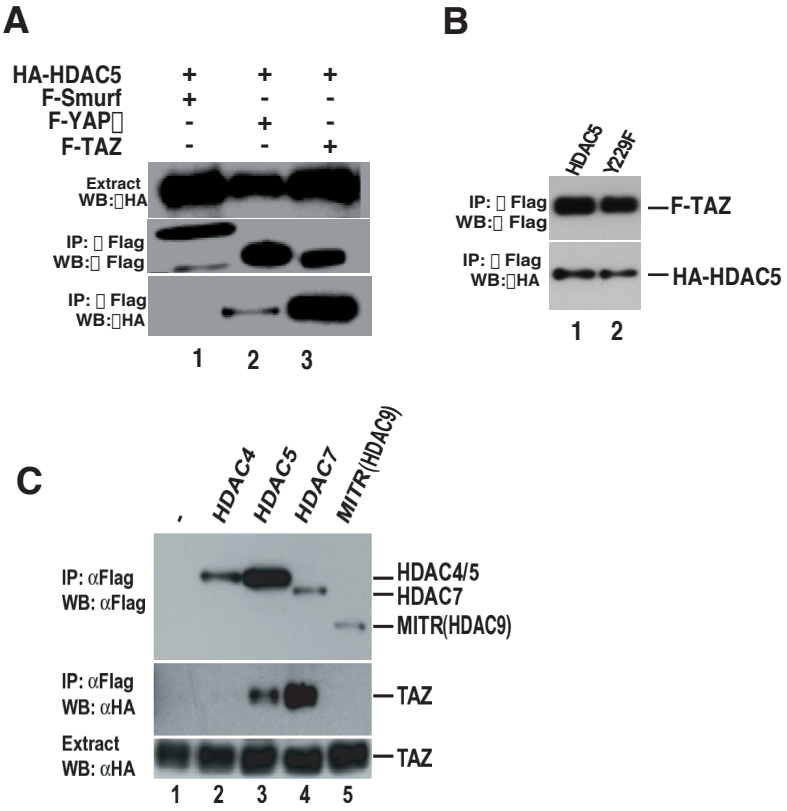
(C) CBP acetylates TAZ and YAPΔ. The experiment was performed as described in panel A. In the presence of sodium butyrate and nicotinamide, strong acetylations of TAZ and YAPΔ are detected (lanes 3 and 4).

(D) Multiple HDACs can deacetylate TAZ. Experiments was performed similarly to those in panel A except that the inhibitor treatments were performed as indicated. Either sodium butyrate or NAD can increase acetylation of TAZ (lanes 1, 2 and 3).

(E) Luciferase assays showing the synergy between TAZ and CBP. 6OSE2-Luc reporter plasmid was used to transfect HEK293 cells along with the plasmids for indicated proteins. β-Gal was used as an internal control. 24 h after tranfection, cells were harvested in lysis buffer followed by determination of Luciferase and β-Gal activities. Co-transfection of TAZ and CBP mediates further increase in the transcriptional activity of Runx2 than either TAZ or CBP alone.

(F) Immunofluorescence microscopy showing the co-localization of TAZ and CBP. Expression plasmids for GFP-TAZ and HA-CBP proteins were transfected into HEK293 cells. 48 h after transfection, cells were stained by anti-HA antibody followed by Alex555 secondary antibody staining. Images were processed using Photoshop. TAZ and CBP colocalize in nuclear dots.

Figure 1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HA-HDAC5
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HDAC4
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Figure 2

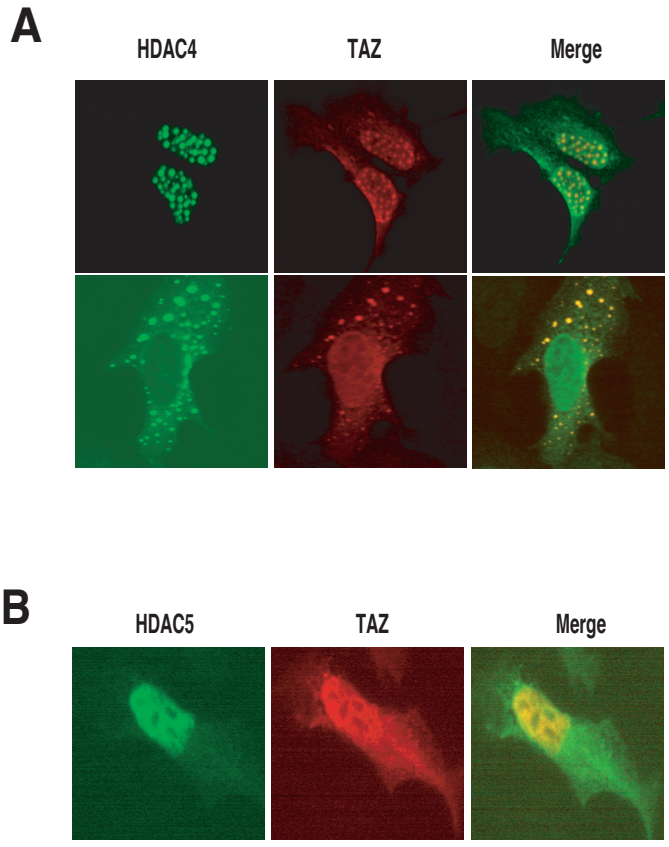

Figure 3

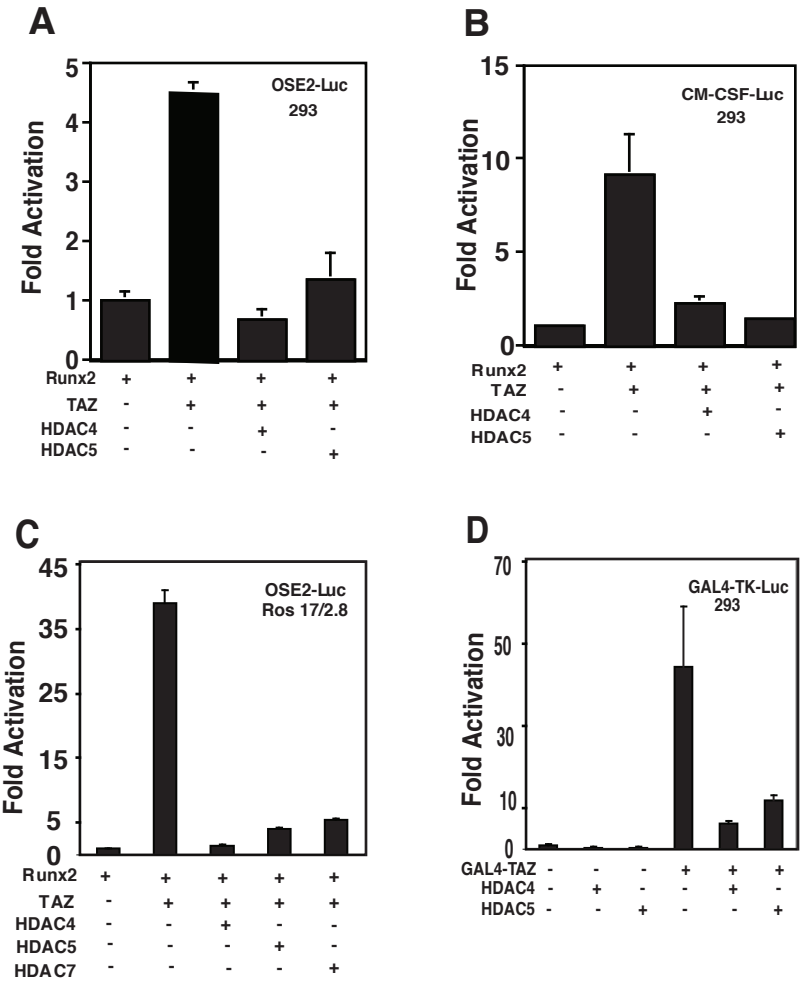
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Figure 4

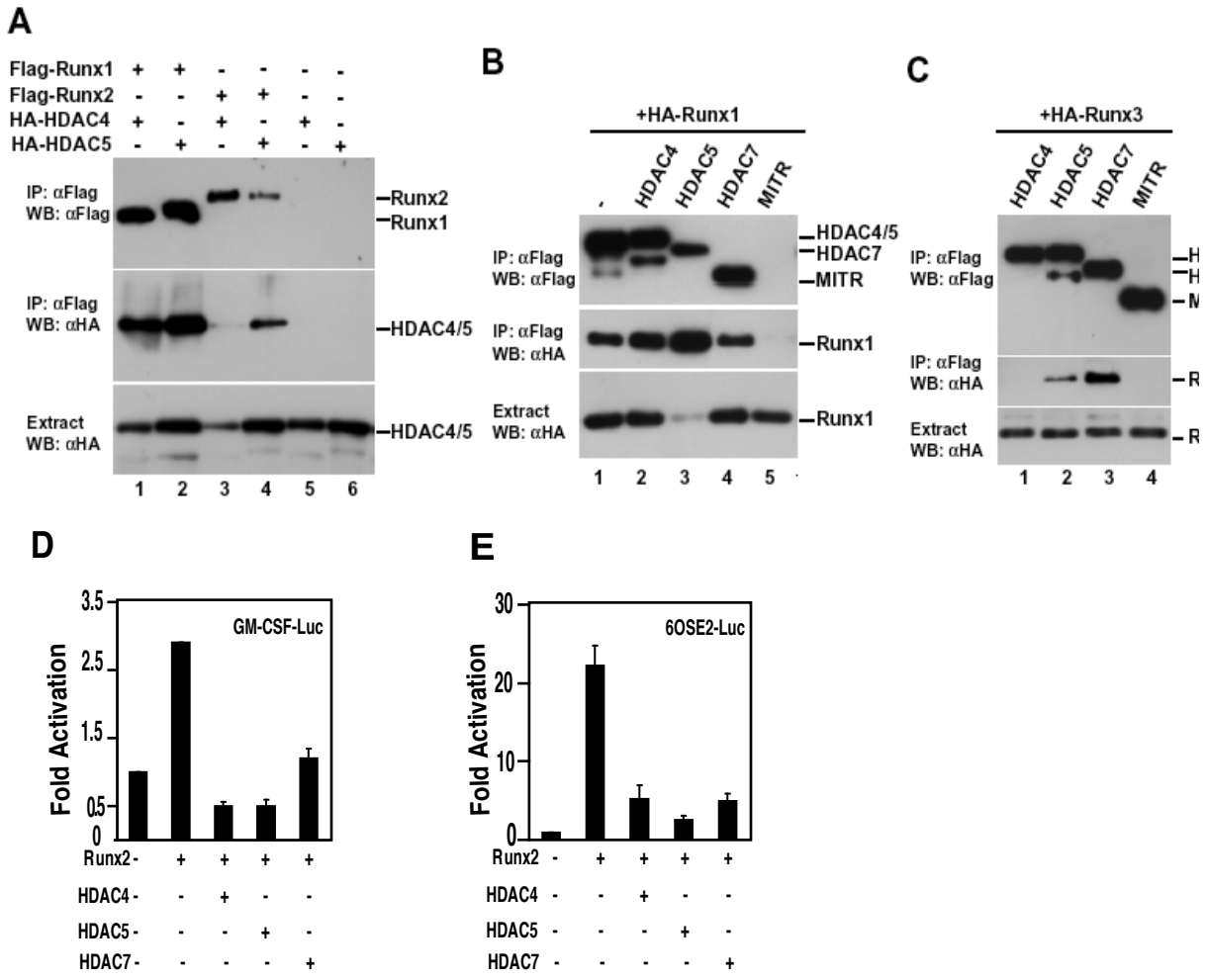
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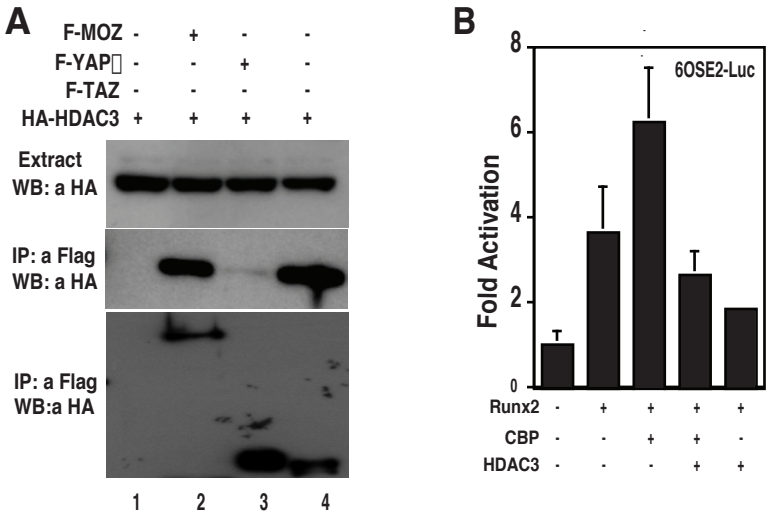
Figure 5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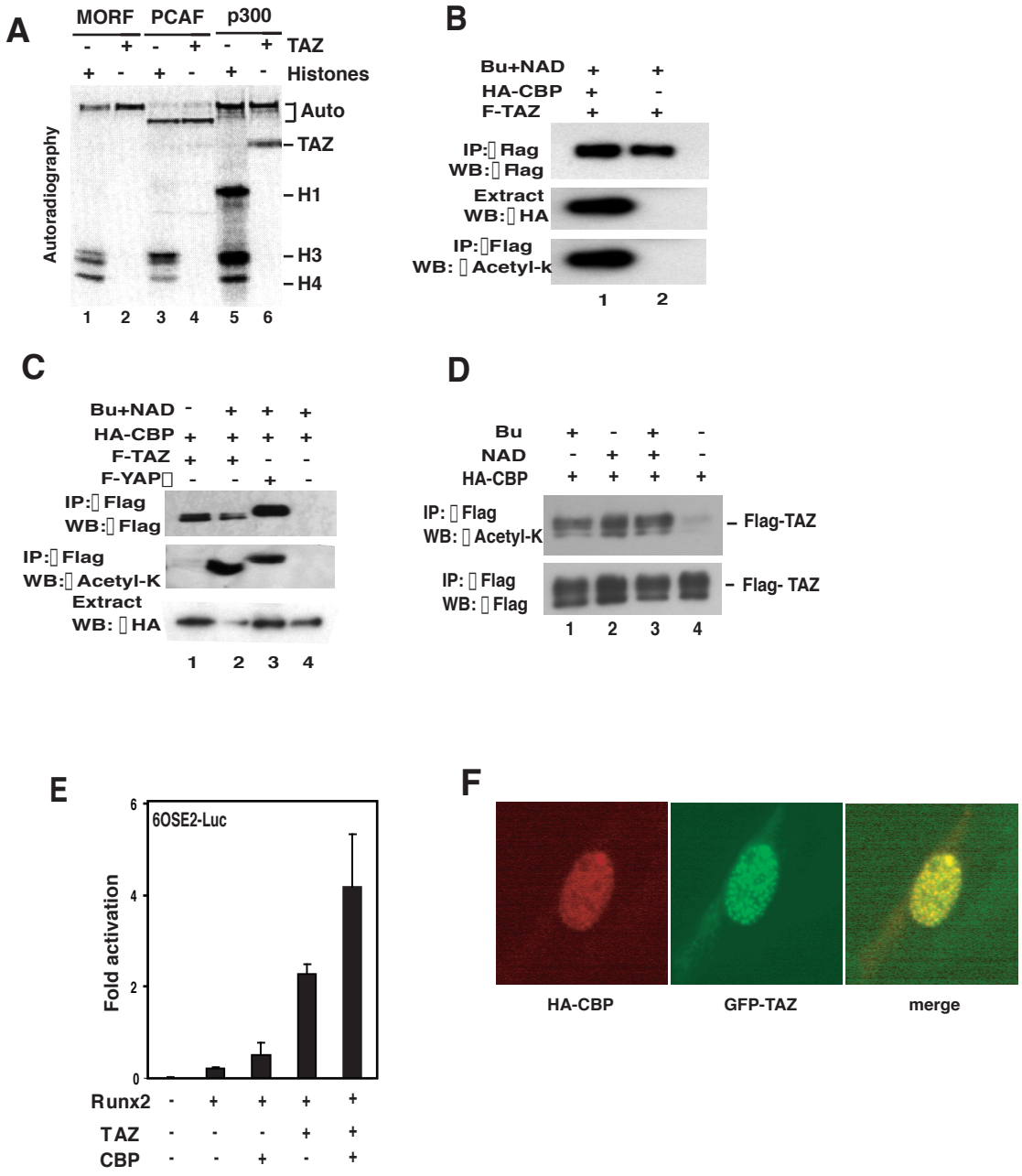
CBP - - + + -
HDAC3 - - - + +

Figure 6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WB: Acetyl-k
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Chapter 4

Regulation of TAZ and YAP by LATS2-mediated multisite phosphorylation

Minghong Xu, Lin Xiao, Serge Gregoire and Xiang-Jiao Yang

Preface

In Chapter 3, we demonstrated that TAZ recruited both repressors and activators to control Runx-dependent transcription. These findings provide good insights in the dual roles of TAZ in transcriptional regulation and are good examples on how transcription is controlled by co-factor recruitment. Following the logic path of the thesis research, we embarked on identifying signaling pathways that regulate the functions of TAZ and YAP. Inspired by the research on *Drosophila* Yorkie, we tested the *hippo*-like pathway in mammalian cells. we found that LATS1 and LATS2, the mammalian homologues of Lats/Warts, are two of the kinases that phosphorylate TAZ and YAP.

Abstract

Although the co-activator activities of TAZ and YAP have been characterized, the signaling pathways that control their functions remain unknown. In light of the study that *Drosophila hippo* phosphorylates and inactivates Yorkie, a *Drosophila* homologue of mammalian YAP, beginning from Oct. 2005, we have been working on elucidating the roles of the *hippo*-like pathway in mammalian cells. Our results show that LATS2 binds to TAZ and YAP. This binding results in phosphorylation of TAZ and YAP both *in vivo* and *in vitro*. The consequence of this phosphorylation is potent transcriptional inactivation. Co-transfection of Mst1 and WW45 strongly potentiates the repression activity of LATS2. Further studies revealed the optimal motif for LATS kinases with the consensus sequence HXRXXS/T, where X is any residue. We found several such sequences in TAZ and YAP. In TAZ, phosphorylation of serine 89 leads to nuclear export and stronger 14-3-3 binding. Phosphorylation of serine 306 does not result in such changes, but leads to potent transcriptional inactivation. Taken together, our studies identified the long sought kinases for TAZ and YAP, and have provided a model of how signaling pathways control the co-factor activities.

Introduction

The regulation of transcription factors and their co-factors by signaling pathways is an everlasting theme of research in understanding most of the fundamental biological processes such as organ development as well as disease formation. The proper development of organisms relies on versatile yet fidel transduction of environmental stimuli to protein modifications which is one of the main effectors in the cell. After completion of the genome project, which has lead to abundant information on the static and linear part of the genome, the more dynamic pictures of how that information is laid out over time and space are awaiting thorough investigation. Our research is to use the mammalian cells. By using various molecular techniques, we try to identify the detailed mechanisms of regulation of transcriptional co-factors by signaling pathways.

Following this line of research, we have focused on the regulations of two transcriptional co-factors named TAZ (transcriptional co-activator with PD Z-binding domain) and YAP (Yes kinase-associated protein). TAZ was first identified in affinity purification using immobilized 14-3-3 proteins (Kanai et al., 2000). Sequence analysis revealed that TAZ contains a WW domain that binds to the PPXY motif. At the carboxyl end, TAZ has a PDZ (post-synaptic density protein PSD95, *Drosophila* tumor suppressor Dlg1, tight junction protein ZO-1) binding motif , suggesting that TAZ binds to PDZ domain-containing proteins (Kanai et al., 2000). TAZ has been shown to interact with Runx2 (Cui et al., 2003), p73 (Strano et al., 2001), and putatively AP2, Krox20, Krox40, MEF2B, NF-E2, Oct-4 (Kanai et al., 2000). Many of these proteins are transcription factors, suggesting that TAZ binds to these proteins and activates transcription. TAZ was

also found to be a co-activator for TEF-1 (Chen et al., 1994; Mahoney et al., 2005), TBX5 (Murakami et al., 2005) and PAX3 (Murakami et al., 2006).

YAP was first identified as Yes kinase-associated protein (Sudol, 1994). Unlike TAZ, YAP possesses an SH3 binding domain and a proline-rich domain. Recent studies showed that YAP is in an amplicon on chromosome 11q22 (Overholtzer et al., 2006). The amplification of YAP is associated with proliferative advantages and cell transformation. More importantly, YAP expression is associated with an EMT (epithelial-mesenchymal transition) phenotype (Overholtzer et al., 2006) . As expected, YAP is also a co-activator for several proteins such as p73 (Strano et al., 2001) and TEF-1 (transcriptional enhancer factor-1), members of the TEF-1 family which bind to MCAT (muscle C, A and T sites) and A/T-rich sites in promoters active in cardiac, skeletal and smooth muscle, placenta, and neural crest (Chen et al., 1994; Mahoney et al., 2005).

Despite the established role of TAZ and YAP as potent co-activators for several transcription factors, there is some evidence suggesting that TAZ and YAP are also co-repressors. An intriguing observation was made when TAZ was co-transfected with peroxisome proliferator-activated receptor γ (PPARγ) (Hong et al., 2005). PPARγ is a transcription factor playing a crucial role in the control of differentiation from mesenchymal to adipose tissue (Chawla et al., 1994; Tontonoz et al., 1994). When TAZ was co-transfected, a decrease was observed in the transcriptional activity of PPARγ with or without ligand. This TAZ-mediated decrease in the transcriptional activity of PPARγ correlated well with the inhibitory role of TAZ in the PPARγ-driven differentiation of mesymchema into adipose tissues. A similar observation has been made with YAP which suppresses Runx2-dependent transcription in ROS17.18 cells (Hong and Yaffe, 2006;

Westendorf, 2006; Zaidi et al., 2004). Although more evidence is needed to fully clarify the inhibitory roles of TAZ and YAP, the concept that a co-activator can be a co-repressor and vice versa is emerging.

The fact that TAZ and YAP serve as either co-activators or co-repressors makes it possible that the conversion between these two distinct roles might be regulated by signaling pathways. Accumulating evidence shows that the binding of co-factors to a transcription factor is a regulated process; co-factors are either released or recruited when certain signaling pathways are activated. For example, CaMKIIΔ signaling releases mSin3a/HDAC co-repressor complex from the MASH1 promoter, and recruits CBP/p300- containing co-activator complex (Ju et al., 2004). Consequently, the question is whether or not such signaling pathways exist regulating TAZ and YAP. A link between YAP and Akt was made when M. Basu tried to identify more Akt substrates using 14-3-3 as bait in affinity purification experiments (Basu et al., 2003). However, this could not be reproduced (Goh, S.L. & Yang, X.J., unpublished data). The crucial discovery that the *hippo* pathway phosphorylates Yorkie, a homologue of YAP (Huang et al., 2005), prompted us to investigate whether mammalian *hippo* kinases regulate TAZ and YAP. Our research, carried out from October 2005 to February 2007, has identified the roles of the *hippo*-like pathway in mammalian cells.

Materials and methods

Cell culture — Human embryonic kidney (HEK293) cells and mouse NIH3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin and streptomycin (Invitrogen).

Mutagenesis of TAZ and YAP mutants — Long-fidelity-based PCR (Roche) was used to generate TAZ and YAP mutants as described in Chapter 2

Co-immunoprecipitation and Western blot — HEK 293 cells were transfected with the expression plasmids using SuperFect (Qiagen). 48 h after transfection, cells were harvested in buffer B150 (20 mM Tris-HCl [pH 8.0], 10% glycerol, 0.15 M KCl, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) plus 50mM NaF. Immunoprecipitation was performed by incubating M2 agarose beads (Sigma) with cell lysates for 2 h at 4°C. The beads were washed 4 times with the same buffer. Bound proteins were eluted with Flag peptide (Sigma). Eluted proteins were mixed with SDS sample buffer and resolved by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane followed by Western blotting as described previously.

Dephosphorylation and in vitro phosphorylation assays — Phosphotase experiments were performed using calf intestine phosphotase (Roche). The expression plasmids for TAZ and/or LATS2 proteins were transfected into HEK293 cells. After 48 h, cells were lysed in buffer B150 (20 mM Tris-HCl [pH 8.0], 10% glycerol, 0.15 M KCl, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors). 6 μl of this cell lysate was mixed with 2 μl

of calf intestine phosphatase (CIP) and 2 μl of CIP buffer. The mixture was incubated at 37°C for 60 min.

For *in vitro* kinase assay, LATS2 were purified from HEK293 cell lysate containing over-expressed F-LATS2 protein with coexpression of Mst1, WW45 and mob1 proteins. F-TAZ was obtained from Sf9 cell lysate. 2 to 4 μl of kinase was mixed with 2 μl of TAZ in kinase buffer containing 25 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM β-glycerophosphate, 1 mM DTT and 0.1 mM ATP. The mixture was incubated at 37°C for 90 min.

Reporter gene assays — SuperFect transfection reagent (Qiagen) was used to transiently transfect a luciferase reporter plasmid (50 to 200 ng) and/or mammalian expression plasmids (50 to 200 ng) into HEK293 or ROS17/2.8 cells. PBluescript KSII (+) was used to normalize the total amount of plasmids used in each transfection, and β-Gal (50 ng) was co-transfected for normalization of transfection efficiency. After 24 to 48 h, cells were lysed and luciferase activity was determined by using D-(L)-luciferin (Boehringer Mannheim) as the substrate. β-Galactosidase activity was measured with Galacto-Light Plus (Tropix) as the substrate. The chemiluminescence from activated luciferin or Galacto-Light Plus was measured on a Luminometer plate reader (Dynex). Each transfection was performed at least three times.

Results

The *hippo* pathway is conserved from *Drosophila* to mammals

Yorkie has been shown to be the target of the *hippo* pathway in *Drosophila* (Huang et al., 2005). Yorkie is the homologue of mammalian YAP (Fig. 1). Compared to YAP and its paralogueTAZ, Yorkie contains the WW domain while lacking the C-terminal PDZ-binding motif. More importantly, the 14-3-3 binding site is well conserved as well as the TEF-1-binding motif (Fig. 1). *Drosophila* Warts/Lats is the key component of the *hippo* pathway. It phosphorylates and inactivates Yorkie (Fig. 2). LATS1 and 2 are mammalian homologues of Warts/Lats. Unlike LATS1 and 2, Warts/Lats lacks the Ubiquitin-associated domain (Fig. 2A). Taken together, sequence comparison of the components of the *hippo* pathway between *Drosoplila* and mammals suggests that YAP and TAZ could be the targets of LATS1 and 2 in mammalian cells.

Physical binding of LATS2 with TAZ and YAP

To prove that, we first tested the interaction between YAP and LATS2. Indeed, in HEK293 cells, YAP bound to LATS2 strongly (Fig. 3A, lane 4). Furthermore, we showed that TAZ, a yap paralogue (Fib. 1B), also bound to LATS2, although less strongly (Fig. 3A, lane 3). Immunoflurescence studies further demonstrated the association of LATS2 with TAZ and YAP in both the nucleus and cytoplasm (data not shown).

Sequence analysis revealed that LATS2 contains a PPXY motif (Fig. 2B), a molecular basis for the interaction with WW domain-containing proteins like TAZ and YAP. We wondered if the PPXY motif could be the binding site between the tested

molecules. We constructed both N- and C-terminal fragments of LATS2 (Fig. 3C). Co-immunoprecipitation showed that both fragments of LATS2 bound to TAZ, indicating that other domains are also involved in the interaction (Fig. 3B). Taken together, these studies establish that LATS2 is the binding partner for TAZ and YAP in mammalian cells.

LATS2 phosphorylates TAZ and YAP

The physical association of TAZ and YAP with LATS2 leads to efficient phosphorylation of TAZ and YAP. Co-transfection of LATS2 with TAZ resulted in a band shift of TAZ (Fig. 4A, lane 3). Calf intestine phosphatase (CIP) treatment confirmed that this band shift was due to phosphorylation (Fig. 4A, lane 4). Similar results were also observed on YAP (Fig. 4B and data not shown). To give evidence that the phosphorylation of TAZ and YAP is the direct effects of LATS2, we performed an *in vitro* phosphorylation assay. LATS2 was purified from HEK293 cell extract containing overexpressed LATS2 with or without Mst1 and WW45. TAZ was purified using a Sf9 cell expression system. LATS2 and TAZ were mixed in the kinase assay buffer. The results showed that LATS2 that had been fully activated by upstream signaling shifted TAZ (Fig. 4B, lanes 3 and 4; Fig. 4C, lanes 1 and 2). In our buffer condition, we did not detect efficient band shift of YAP *in vitro* (Fig. 4C, lanes 3 and 4).

Furthermore, we wanted to give further proof of LATS2-mediated phosphorylation of TAZ and YAP by identifying the substrate serine residues. We performed *in vitro* and *in vivo* phosphorylation studies using serine mutants of TAZ and YAP. A previous study has shown that serine 89 of TAZ and serine 127 of YAP are the

sites for phosphorylation, leading to their cytoplasmic retention (Kanai et al., 2000). We investigated if LATS2 could also act upon these serines. One dramatic effect occured when LATS2 and TAZ were co-expressed. The expression levels of both molecules increased significantly (Fig. 4D). Using this effect, we were able to detect that the serine 89 mutant of TAZ (S89A) behaved differently from wild type, indicating the serine 89 is under control of LATS2. Indeed, LATS2, in the presence of Mst1 and WW45, drove TAZ out of the nucleus (data not shown) and resulted in stronger 14-3-3 association (Fig. 5G). More importantly, sequence analysis revealed that there are several similar serine-containing motifs in TAZ. One of them is serine 306. The S306A mutant of TAZ showed no band shift, no expression level changes upon co-expression with LATS2, demonstrating that serine 306 of TAZ is also an important target of LATS2 (Fig. 4D). We identified four such serines in TAZ and five in YAP. In TAZ, the other two are serines 66 and 117. Mutation of these serines does not change the band shift pattern upon co-expression with LATS2 (Fig. 4E).

So far, our mutation studies strongly suggest that TAZ and YAP are two targets of phosphorylation by LATS2. In order to gain direct evidence that these identified serines are phosphorylated by LATS2, we generated anti-pS89 and anti-pS306 antibodies for TAZ. As shown by anti-pS89 antibody, serine 89 is constitutively phosphorylated in HEK293 cells (Fig. 4F). The presence of LATS2 did not significantly change the phosphorylation state of it. Phosphorylation of serine 306, however, increased dramatically when LATS2 and its upstream activators were present (Fig. 4F). These results give direct evidence that LATS2 acts upon TAZ and YAP by targeting specific serine residues.

Functional consequences of phosphorylation of serine 89 in TAZ (serine 127 in YAP) by LATS2

As shown by our previous results, serine 89 in TAZ and 127 in YAP are two major sites phosphorylated by LATS2. These serines bind to 14-3-3, leading to nuclear export of TAZ (Kanai et al, 2000) and YAP (Basu et al, 2003). We investigated whether the presence of LATS2 could result in such changes. The co-immunoprecipitation showed that the binding between TAZ and 14-3-3 was much stronger when LATS2 and its upstream inputs were present. (Fig. 5A, lane 4 in IP samples). Similar results were obtained for YAP (Fig. 5A, lanes 6 and 7 in IP samples), co-expression of Mst1 and WW45 with LATS2 led to strong binding between YAP and 14-3-3. This binding led to nuclear export of YAP (Fig. 5B). The serine 127 mutation of YAP abolished the nuclear export (Fig. 5B). Taken together, serine 89 in TAZ and serine 127 in YAP are targets of LATS2, phosphorylation of which leads to efficient nuclear export of TAZ and YAP.

LATS2 represses the co-activator activities of TAZ and YAP

We have demonstrated that LATS2 binds to and phosphorylates TAZ and YAP. We sought to understand the functional consequences. TAZ is known as a co-activator for Runx family proteins (Cui et al., 2003). Through the WW domain, TAZ binds to the PPXY motif of Runx and activates transcription. In our experiments, LATS2 potently repressed the activities of TAZ and YAP (Fig. 5A, 5B), while the kinase dead mutant (LATS2KD) although retained some effects, but showed much less potency, (Fig. 5C).

This suggests that the kinase activity of LATS2 is the major factor repressing the co-activator activity of TAZ. The residual effect of LATS2KD could be due to competition of LATS2KD with Runx proteins since they both contain PPXY motif. Indeed, the N-terminal truncation mutant of LATS2 with the PPXY motif can still repress TAZ-dependent co-activation (data not shown). To demonstrate the direct effect of LATS2 on the co-activator activities of TAZ and YAP, we generated TAZ and YAP fused with GAL4-DBD. Using GAL4-TK-Luc as reporter, we showed that, in the presence of Mst1 and WW45, LATS2 repressed the co-activator activities of TAZ and YAP potently (Fig. 5D, 5E). Strikingly, the TAZ S89A/S306A failed to respond to LATS2-mediated transcriptional repression, which is consistent with that the inhibitory effect of LATS2 is through phosphorylation of identified serine residues (Fig. 5F).

Identification of a novel consensus sequence for *hippo*-like kinase in mammalian cells

The residues adjacent to the serines 89 and 306 in TAZ are conserved among different species (Fig. 1), suggesting their roles in determining the substrate specificity. By means of band shift and reporter gene assays (as exemplified in Fig. 6B), we delineated HXRXXS/T as the consensus sequence, where X is any residue (Fig. 6A). Mutation of histidine (H) and arginine (R) to alanine makes this motif unfavorable for LATS2 phosphorylation. Residues similar in chemical properties to H or R can replace them (Fig. 6). Interestingly, not all serines and their adjacent residues identified in this way function the same. For example, LATS2 still exerted potent transcriptional repression on S66A and H61A mutations of TAZ (data not shown).

The role of HDACs in the LATS2-related repression of co-activation by TAZ and YAP

In Chapter 3, I have shown that TAZ recruited HDACs to serve as a repressor. We wondered if HDACs played roles in LATS2-mediated repression. In the co-immunoprecipitation study, we showed that LATS2 bound to HDAC3 strongly (Fig. 7A). More importantly, TAZ, HDAC3 and LATS2 formed a trimeric protein complex when co-expressed (Fig. 7A). We also tested HDAC4. As shown in our previous chapter, HDAC4 barely binds to TAZ in our buffer condition. The presence of LATS2, however, induced stronger association between TAZ and HDAC4 (Fig. 7B). These three proteins also form a complex. These data demonstrate that the presence of LATS2 induces a TAZ and HDACs-containing repressor complex that leads to transcriptional repression.

Discussion

We identified the kinase for TAZ and YAP in mammalian cells. The co-immunoprecipitation studies showed strong and stable binding between LATS2 and YAP or TAZ. As shown by both *in vivo* and *in vitro* phosphorylation assays, this physical interaction led to efficient phosphorylation of TAZ and YAP. In turn, this phosphorylation event resulted in their transcriptional inactivation. The presence of the upstream elements of the *hippo*-like pathway leads to further inactivation. These findings were further proved by the identification of the phosphorylation sites in TAZ and YAP, which lead to a new substrate consensus sequence for LATS. Taken together, these results establish the role of the *hippo*-like pathway in regulating the co-activator functions of TAZ and YAP (Fig. 7).

When the manuscript was in preparation, similar studies were published by other groups (Dong et al., 2007; Hao et al., 2008; Lei et al., 2008; Zhang et al., 2008; Zhao et al., 2007). The findings confirmed that TAZ and YAP are the authentic targets of LATS kinases in mammalian cells and made a significant step towards understanding the mechanisms of mammalian organ size control. But many questions are also generated by these exciting studies.

How does phosphorylation of TAZ and YAP result in transcriptional inactivation? Our previous study showed that TAZ recruits HDACs to serve as a co-repressor (Chapter 3). We reasoned that the phosphorylation events that occurred in TAZ and YAP could create docking sites for more potent recruitment of HDACs. Indeed, we found that LATS2 bound to HDAC3 strongly. Furthermore, we showed that in the presence of LATS2, the binding between HDAC4 and TAZ was much stronger, indicating that a

potent co-repressor complex was induced when LATS2 signaling is present. Another possible explanation is that the phosphorylation strengthens the association between TAZ and YAP with TRCP (Tian et al., 2007).

The marked changes of TAZ function are not only limited to its co-activator activities. A remarkable phenomenon happened when LATS2 and TAZ were coexpressed: the expression level of both TAZ and LATS2 increased dramatically, which is not the case when YAP was co-transfected with LATS2 nor when the consensus sequence of TAZ was mutated. It is clear to us that TAZ and LATS2 "talk" to each other although their "language" is unknown. The fact that phosphorylation modification is essential makes us wonder if other protein modifications play roles in their "dialogue". Indeed, we have found that LATS2 is acetylated and sumoylated (data not shown). How each modification affects others and how these modifications orchestrate remain to be explored.

We have identified the consensus sequence for LATS2 kinase. TAZ and YAP harbor several such sequence motifs. Interestingly, the consequences of phosphorylation of each motif are different. In TAZ, phosphorylation of serine 89 leads to cytoplasmic retention (data not shown), while it has minimal effects on the transcriptional activity. Phosphorylation of serine 306, however, leads to complete transcriptional inactivation without any effects on the nuclear localization. The effect of phosphorylation of serines 66 and 117 are under investigation. Multiple site phosphorylations of TAZ and YAP could potentially create more binding site for HDACs, leading to efficient transcriptional repression.

The structural similarity between LATS1 and LATS2 suggests similar functions. It has been found that LATS1 also targets YAP (Hao et al., 2008). However, we did not observe the effect of LATS1 on the regulation of TAZ, suggesting the disparity of these two kinases (data not shown). Indeed, the gene knockout studies have shown the functional difference. LATS2 knockout is embryonic lethal, but MEFs derived from LATS2 knockout animals display many profound defects in mitosis (McPherson et al., 2004). LATS1 knockout mice are viable but develop soft tissue tumors at later stages. The mice also have pituitary dysfunction (St John et al., 1999). Why do close relatives have divergent functions remains a mystery.

References

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*, 11-23.

Chawla, A., Schwarz, E. J., Dimaculangan, D. D., and Lazar, M. A. (1994). Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology *135*, 798-800.

Chen, Z., Friedrich, G. A., and Soriano, P. (1994). Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes Dev *8*, 2293-2301.

Cui, C. B., Cooper, L. F., Yang, X., Karsenty, G., and Aukhil, I. (2003). Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. Mol Cell Biol *23*, 1004-1013.

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*, 1120-1133.

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*, 5496-5509.

Hong, J. H., Hwang, E. S., McManus, M. T., Amsterdam, A., Tian, Y., Kalmukova, R., Mueller, E., Benjamin, T., Spiegelman, B. M., Sharp, P. A.*, et al.* (2005). TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. Science *309*, 1074-1078.

Hong, J. H., and Yaffe, M. B. (2006). TAZ: a beta-catenin-like molecule that regulates mesenchymal stem cell differentiation. Cell Cycle *5*, 176-179.

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*, 421-434.

Ju, B. G., Solum, D., Song, E. J., Lee, K. J., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2004). Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway. Cell *119*, 815-829.

Kanai, F., Marignani, P. A., Sarbassova, D., Yagi, R., Hall, R. A., Donowitz, M., Hisaminato, A., Fujiwara, T., Ito, Y., Cantley, L. C., and Yaffe, M. B. (2000). TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. Embo J *19*, 6778-6791.

Lei, Q. Y., Zhang, H., Zhao, B., Zha, Z. Y., Bai, F., Pei, X. H., Zhao, S., Xiong, Y., and Guan, K. L. (2008). TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. Mol Cell Biol *28*, 2426-2436.

Mahoney, W. M., Jr., Hong, J. H., Yaffe, M. B., and Farrance, I. K. (2005). The transcriptional co-activator TAZ interacts differentially with transcriptional enhancer factor-1 (TEF-1) family members. Biochem J *388*, 217-225.

McPherson, J. P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak-Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J.*, et al.* (2004). Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. Embo J *23*, 3677-3688.

Murakami, M., Nakagawa, M., Olson, E. N., and Nakagawa, O. (2005). A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. Proc Natl Acad Sci U S A *102*, 18034-18039.

Murakami, M., Tominaga, J., Makita, R., Uchijima, Y., Kurihara, Y., Nakagawa, O., Asano, T., and Kurihara, H. (2006). Transcriptional activity of Pax3 is co-activated by TAZ. Biochem Biophys Res Commun *339*, 533-539.

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*, 12405-12410.

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*, 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*, 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*, 2145-2152.

Tian, Y., Kolb, R., Hong, J. H., Carroll, J., Li, D., You, J., Bronson, R., Yaffe, M. B., Zhou, J., and Benjamin, T. (2007). TAZ promotes PC2 degradation through a SCFbeta-Trcp E3 ligase complex. Mol Cell Biol *27*, 6383-6395.

Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell *79*, 1147-1156.

Westendorf, J. J. (2006). Transcriptional co-repressors of Runx2. J Cell Biochem *98*, 54-64.

Yang, X. J., and Gregoire, S. (2006). A recurrent phospho-sumoyl switch in transcriptional repression and beyond. Mol Cell *23*, 779-786.

Zaidi, S. K., Sullivan, A. J., Medina, R., Ito, Y., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. (2004). Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. Embo J *23*, 790-799.

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

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L.*, et al.* (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev *21*, 2747-2761.

Figure 1

Domains and motifs are conserved among TAZ, YAP and Yorkie

(A) Comparison of Mammalian YAP and TAZ with *Drosophila* Yorkie. Mammalian YAP contains two WW domains, an SH3-binding domain, and a PDZ-binding motif. Serine 127 is the site for binding to 14-3-3. TAZ shares 45% sequence identity to YAP. Unlike YAP, TAZ contains only one WW domain. *Drosophila* Yorkie lacks the PDZ- and SH3-binding motifs. The sequence homology is shown on the right side

(B) Sequence alignment of human TAZ and *Drosophila* Yorkie (Yrk). The alignment was generated by using the two sequence comparison tool on the BLAST website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?1). The TAZ sequence (GenBank accession number NP_056287) was aligned to that of Yorkie (AAZ42161), with the resulting alignment copied and pasted to Adobe Illustrator for further processing. Known and potential motifs for protein interaction, as well as the tandem WW domains, are indicated with solid lines. The 14-3-3 binding site was manually edited to achieve a better alignment. Like the putative TRCP binding motif, this site is much more conserved than the surrounding regions and thus forms a small sequence 'conservation island' (Yang and Gregoire, 2006).

(C) Sequence comparison of human TAZ and YAP. The alignment was generated and processed as above. The TAZ sequence was aligned to that of YAP (GenBank accession number XP_001151402).

Figure 2

The *hippo* pathway is conserved from *Drosophila* to mammals

(A) Diagrammatic illustration of *Drosophila* Warts/Lats with LATS1 and 2. LATS1 and 2 are highly homologous. Both contain a kinase domain, an ubiquitin-associated domain and a highly conserved PPXY motif. Unlike LATS1 and 2, Warts lacks the ubiquitin-associated domain. The homology of the kinase domain is shown on the right side.

(B) Summary of the *hippo* pathways in both *Drosophila* and mammals. In *Drosophila*, the *hippo* pathway is well characterized. Merlin and Expanded activate downstream kinases, leading to inactivation of Yorkie. In mammals, however, it is unclear about the initial inputs that result in activation of *hippo*-like pathways. Another important but unaddressed question is that whether YAP is under control of *hippo*-like pathways in mammals.

Figure 3

LATS2 interacts with TAZ and YAP

(A) Both TAZ and YAP interact with LATS2. Expression plasmids for indicated proteins were transfected into HEK293 cells. 48 h later, cells were harvested in buffer B150 (20 mM Tris-HCl [pH 8.0], 10% glycerol, 0.15 M KCl, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) plus 50mM NaF. Co-immunoprecipitation was performed by incubation of M2 agarose beads with cell lysates for 2 h at 4°C. The beads were washed 4 times with the same buffer. Bound proteins were eluted with Flag peptide. Eluted proteins were mixed with the SDS sample buffer and resolved by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane for Western blotting. Both TAZ and YAP interact with LATS2 (lanes 3 and 4).

(B) Both N- and C- terminal fragments interact with TAZ. Co-immunoprecipitation was performed as described in panel A. The C-terminal fragment of LATS2 shows no kinase activity since no band shift of TAZ occurs.

(C) Summary of the TAZ-binding abilities of LATS2 and its fragments. The intensity of the binding is indicated by the number of "+". The N-terminal mutant binds to TAZ more strongly than full length LATS2.

Figure 4

LATS2 phosphorylates TAZ both *in vivo* and *in vitro*

(A) LATS2 phosphorylates TAZ *in vivo*. Expression plasmids for indicated proteins were transfected into HEK293 cells. 48 h later, cells were lysed in buffer B150. Calf intestine phosphatase (CIP) treatment was performed (see Materials and Methods). Western blot was performed using anti-HA antibody. Co-expression of TAZ and LATS2 leads to a band shift of TAZ (lane 3). CIP treatment diminishes the upper band significantly (lane 4).

(B) LATS2 phophorylates YAP *in vivo*. F-YAP and HA-LATS2 were co-transfected into HEK293 cells as indicated. 48 h later, cells were lysed in buffer K150 (20 mM sodium phosphate, pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors) followed by western blotting. LATS2 causes a band shift of YAP, indicating phosphorylation.

(C) Fully activated LATS2 by Mst1 and WW45 phosphorylates TAZ *in vitro*. LATS2 was purified from HEK293 cells overexpressing LATS2 proteins with or without Mst1 and WW45 (lane 1: no kinase; 2: LATS2 alone; 3: 2 μl LATS2 co-expressed with MST and WW45; 4: 4 μl LATS2 co-expressed with Mst1 and WW45). Only LATS2 co-expressed with Mst1 and WW45 can cause band shift of TAZ (lanes 4 and 5), while LATS2 purified alone cannot (lane 3).

(D) *In vitro* Kinase assay using affinity-purified LATS2 co-expressed with Mst1 and WW45. The experiments were performed as described in panel B. The substrates are TAZ (lanes 1 and 2), YAP (lanes 3 and 4), YAPm (lanes 5 and 6). YAPm is a mutant containing five serine mutations. They are S61A, S109A, S127A, S164A, S347A. The

products were blotted with either anti-Flag or anti-pS89 antibody. LATS2 causes band shift of TAZ, not YAP or YAPm.

(E) Phosphorylation of TAZ and its mutant by LATS2. Expression plasmids were transfected into HEK293 cells as indicated. 48 h later, cells were lysed in buffer K150 followed by Western blotting. Note the increased expression level and band shift of wild type TAZ, which is not observed when TAZ mutants are co-expressed with the LATS2.

(F) Phosphorylation of serines 66, 89, 117 and 306 in TAZ by LATS2 *in vivo*. Experiments were performed as described in panel D. Mutation of serine 66 and 117 did not change the expression pattern of TAZ.

(G) Phosphorylation of serines 89 and 306 of TAZ by LATS2. Expression plasmids for indicated proteins were transfected into HEK293 cells. 48 h later, cells were lysed in buffer K150 followed by Western blot with anti-pS89 and anti-pS306 antibodies. There is a significant increase of phosphorylation of serine 306 in TAZ, especially when Mst1 and WW45 are present (lanes 1, 2 and 3). These antibodies can also recognize corresponding serines in YAP because of sequence homology. Serines 89 and 306 of TAZ correspond to serines 127 and 347 in YAP, respectively. (lanes 4, 5 and 6).

Figure 5

The consequences of phosphorylation of serine 89 in TAZ and serine 127 in YAP

(A) Expression plasmids for indicated proteins were transfected into HEK293 cells. 48 h later, co-immunoprecipitation was performed as described previously. Both TAZ and YAP interact with 14-3-3 (lanes 2 and 5 in the IP samples). The interaction between YAP and 14-3-3 is enhanced by the presence of LATS2 (lane 6 in the IP lanes). Mst1 and WW45 stimulate this binding even further (lane 7 in the IP lanes).

(B) LATS2 promotes nuclear export of YAP. YAP_ was expressed in HEK293 cells as GFP-tagged protein for live green fluorescence microscopy. HEK293 cells were transiently transfected with the expression plasmids and images were taken after 18 h. *Right*, LATS2, Mst1 and WW45 were co-expressed with YAP_. YAP S127A was also transfected for analysis. Arrows indicate cells with cytoplasmic accumulation of YAP_.

Figure 6

LATS2 represses the co-activator activities of TAZ and YAP

(A) 6OSE2-Luc was used as reporter. In this reporter, the luciferase gene is under control of six repeats of Runx2/Cbfa1 responsive element in osteocalcin promoter. Expression plasmids for indicated proteins were transfected into HEK293 cells. β-Gal plasmid was also transfected as an internal control. 48 h later, cells were lysed in luciferase buffer. Luciferase and β-Gal activities were determined. LATS2 represses the co-activation mediated by TAZ.

(B) GM-CSF-Luc reporter and plasmids for indicated proteins were transfected into HEK293 cells. The luciferase assay was performed as described in panel A. LATS2 repressed the co-activator activities of TAZ and YAP on GM-CSF-Luc reporter.

(C) Transfection and the luciferase assay were performed similarly as described in panel A. Kinase dead mutant fails to repress the co-activity of TAZ.

(D) GAL4-TK-Luc reporter was used. The luciferase assay was performed as described in panel A. LATS2 alone shows no repressive activity on TAZ. The co-transfection of Mst1 and WW45 with LATS2 represses the co-activity of TAZ potently.

(E) GAL4-TK-Luc reporter was co-transfected with plasmids for indicated proteins. The luciferase assay was performed as described in panel A. LATS2 alone shows no repressive activity on YAP. The co-transfection of Mst1 and WW45 with LATS2 repress the co-activity of YAP potently.

(F) LATS2 fails to repress the co-activator activity of S89A/S306A mutant of TAZ. Expression plasmids for indicated proteins were transfected into HEK293 cells. β-Gal plasmid was also transfected as an internal control. 24 h later, cells were lysed in

luciferase buffer. Luciferase and β-Gal activities were determined. Both wild type and S89A/S306A mutant of TAZ activate transcription potently. The presence of LATS2 and its upstream kinases fails to inhibit the activity of this mutant TAZ.

(G) Expression plasmids for indicated proteins were transfected into HEK293 cells. 48 h later, co-immunoprecipitation was performed as described previously. Both TAZ and YAP interact with 14-3-3 (lanes 2 and 5). The interaction between YAP and 14-3-3 is enhanced by the presence of LATS2 (lane 6). Mst1 and WW45 stimulate this binding even further (lane 7).

Figure 7

Delineation of the phosphorylation substrate sequence in TAZ

(A) Delineation of the consensus sequence of phosphorylation in TAZ. The residues of TAZ from 299 to 307 are shown. Each indicated mutation was made. The mutant was used in band shift study and luciferase assays. The results are summarized on the right side. Based on these results, the consensus sequence is delineated as HXRXXS/T, where X is any residue.

(B) An example of band shift assay demonstrates that the adjacent residues are essential for LATS2-mediated phosphorylation of TAZ. The plasmids for indicated proteins were transfected into HEK293 cells. 48 h later, cells were lysed in buffer K150. Anti-HA antibody was used in the Western blot. Co-expression of LATS2 with wild type TAZ causes a band shift, indicating a phosphorylation event. Mutant TAZ proteins show no such band shifts.

Figure 8

HDACs were recruited by TAZ upon LATS2 simulation

(A) Expression plasmids for indicated proteins were transfected into HEK293 cells. 48 later, cells were lysed in buffer K150. Co-immunoprecipitation experiments were performed as described in figure 2. HDAC4 shows very weak binding with TAZ in our buffer condition (lane 4). The presence of kinase leads to stronger association between HDAC4 and TAZ (lane 2).

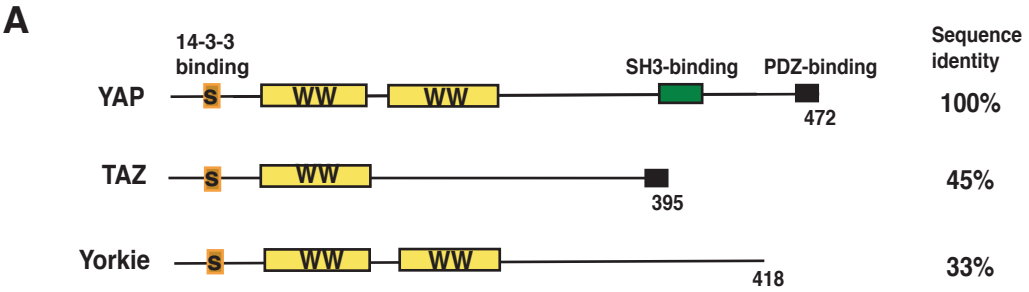
(B) HDAC3, TAZ and LATS2 form trimeric complex. Expression plasmids for indicated proteins are transfected into HEK293 cells. 48 h later, cells were lysed in BK150. Co-immunoprecipitation experiments were performed as described in figure 2. TAZ shows strong binding to HDAC3 (lane 1). LATS2 and HDAC3 are pulled down together with TAZ, indicating that these three proteins form a complex (lane 2).

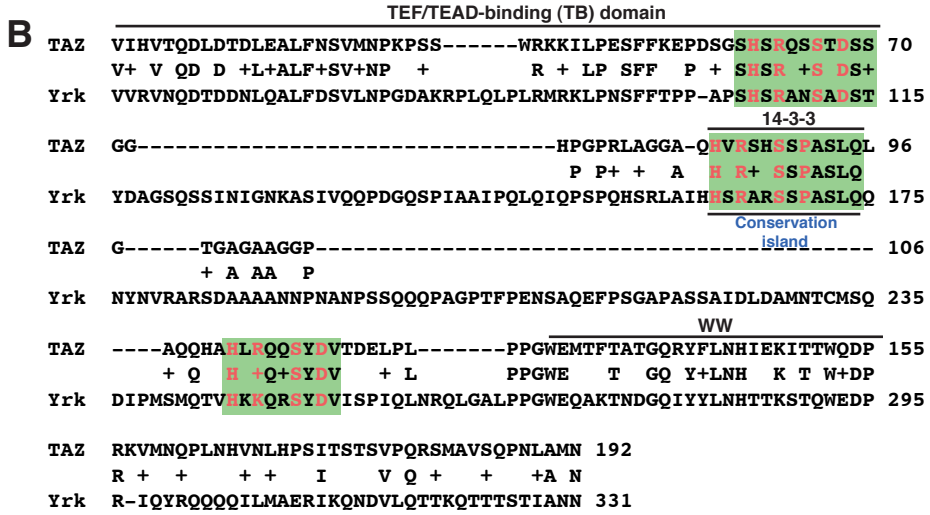
Figure 9

Summary of serines phosphorylated by LATS2 and their functional consequences

TAZ contains four serines conformed to the consensus sequence HXRXXS/T. They are serines 66, 89, 117 and 306. Serine 89 phosphorylation leads to stronger 14-3-3 association and TAZ nuclear export. Serine 306 phosphorylation by LATS2 results in transcriptional inactivation of TAZ, probably due to recruitment of HDACs and $SCF^{\beta\text{-}TRCP}$, an E3 ubiquitin ligase. The exact functions of phosphorylation at serines 66 and 117 by LATS2 are unknown.

Figure 1


A
14-3-3 binding
SH3-binding
PDZ-binding
Sequence identity
YAP
S
WW
WW
472
100%
TAZ
S
WW
395
45%
Yorkie
S
WW
WW
418
33%


B
TEF/TEAD-binding (TB) domain
14-3-3
Conservation island
WW

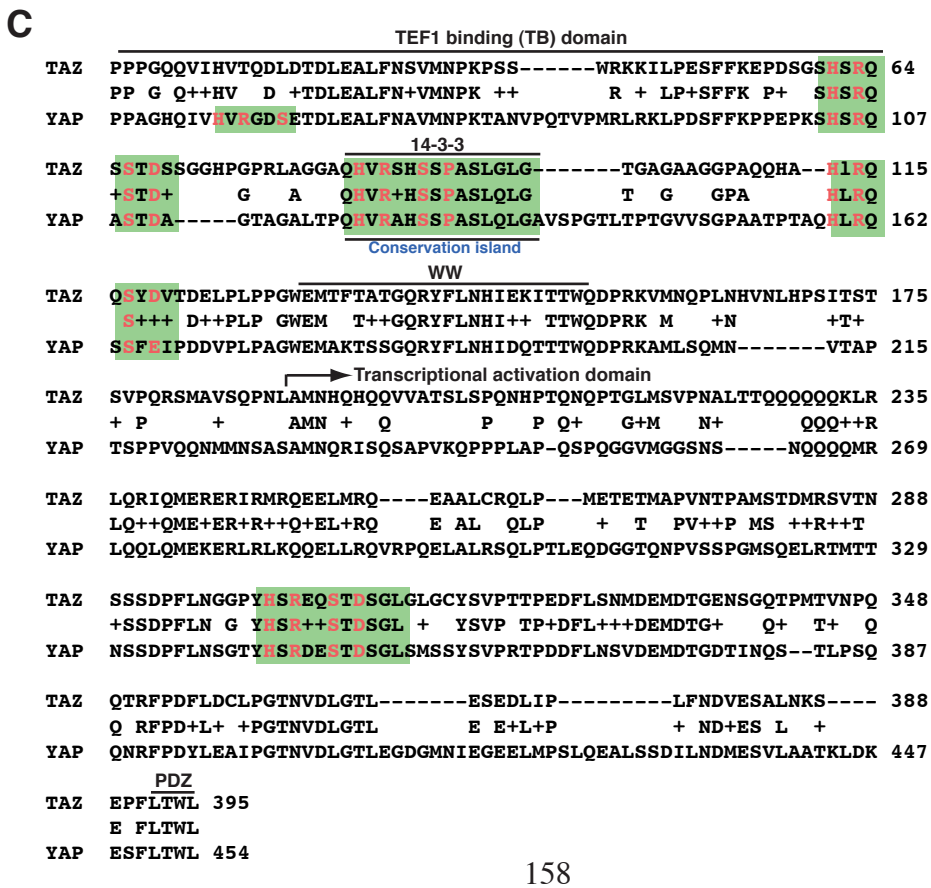
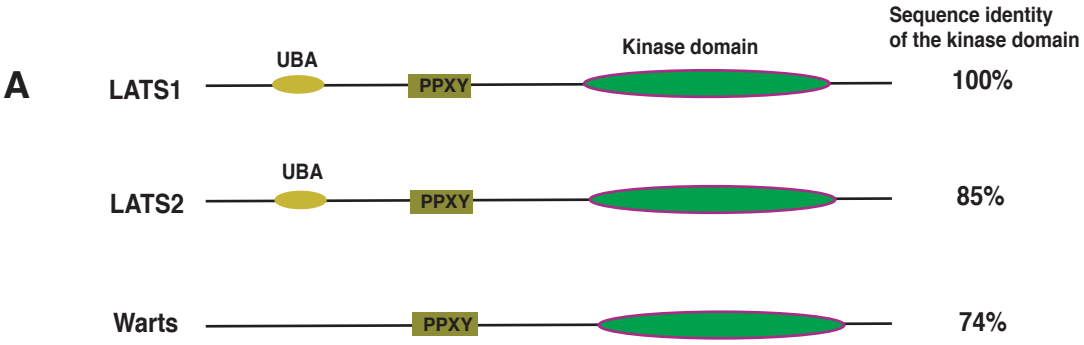

C
TEF1 binding (TB) domain
14-3-3
Conservation island
WW
Transcriptional activation domain
PDZ

Figure 2


A
Sequence identity
of the kinase domain
Kinase domain
UBA
LATS1
PPXY
100%
UBA
LATS2
PPXY
85%
Warts
PPXY
74%

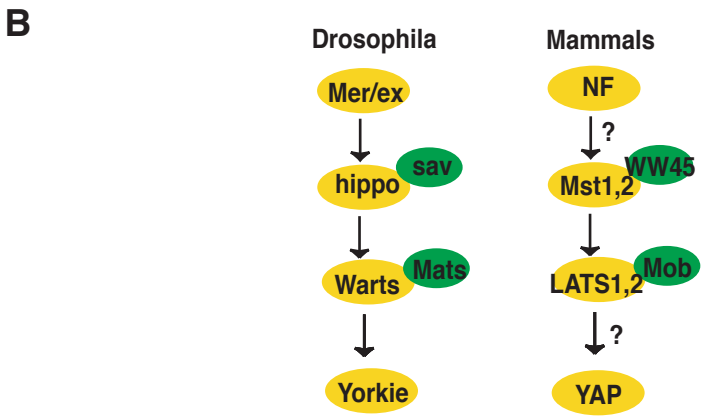

B
Drosophila
Mammals
Mer/ex
sav
hippo
Mats
Warts
Yorkie
NF
?
WW45
Mst1,2
Mob
LATS1,2
?
YAP

Figure 3

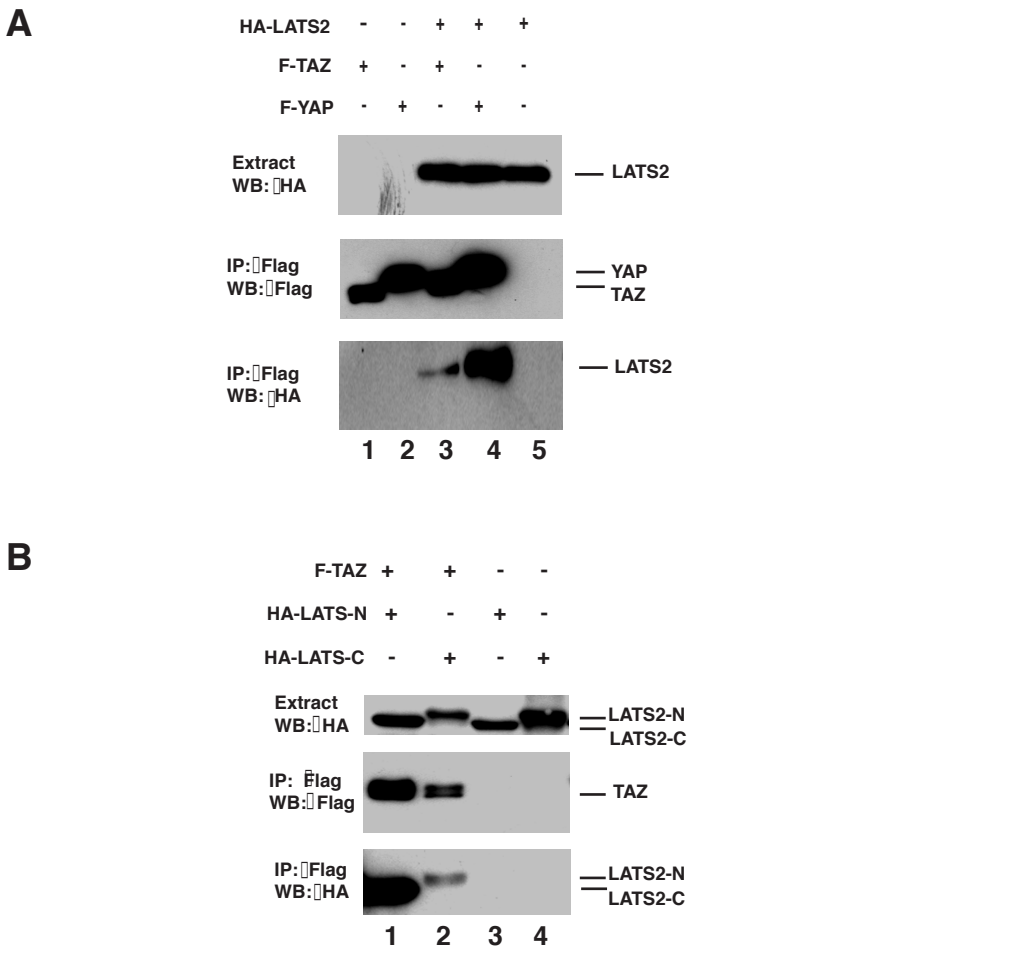

A
HA-LATS2 - - + + +
F-TAZ + - + - -
F-YAP - + - + -
Extract
WB: HA
LATS2
IP: Flag
WB: Flag
YAP
TAZ
IP: Flag
WB: HA
LATS2
1 2 3 4 5
B
F-TAZ + + - -
HA-LATS-N + - + -
HA-LATS-C - + - +
Extract
WB: HA
LATS2-N
LATS2-C
IP: Flag
WB: Flag
TAZ
IP: Flag
WB: HA
LATS2-N
LATS2-C
1 2 3 4
C
TAZ binding
LATS2-FL
PPXY
+ +
LATS2-N
PPXY
+ + +
LATS2-C
+

Figure 4

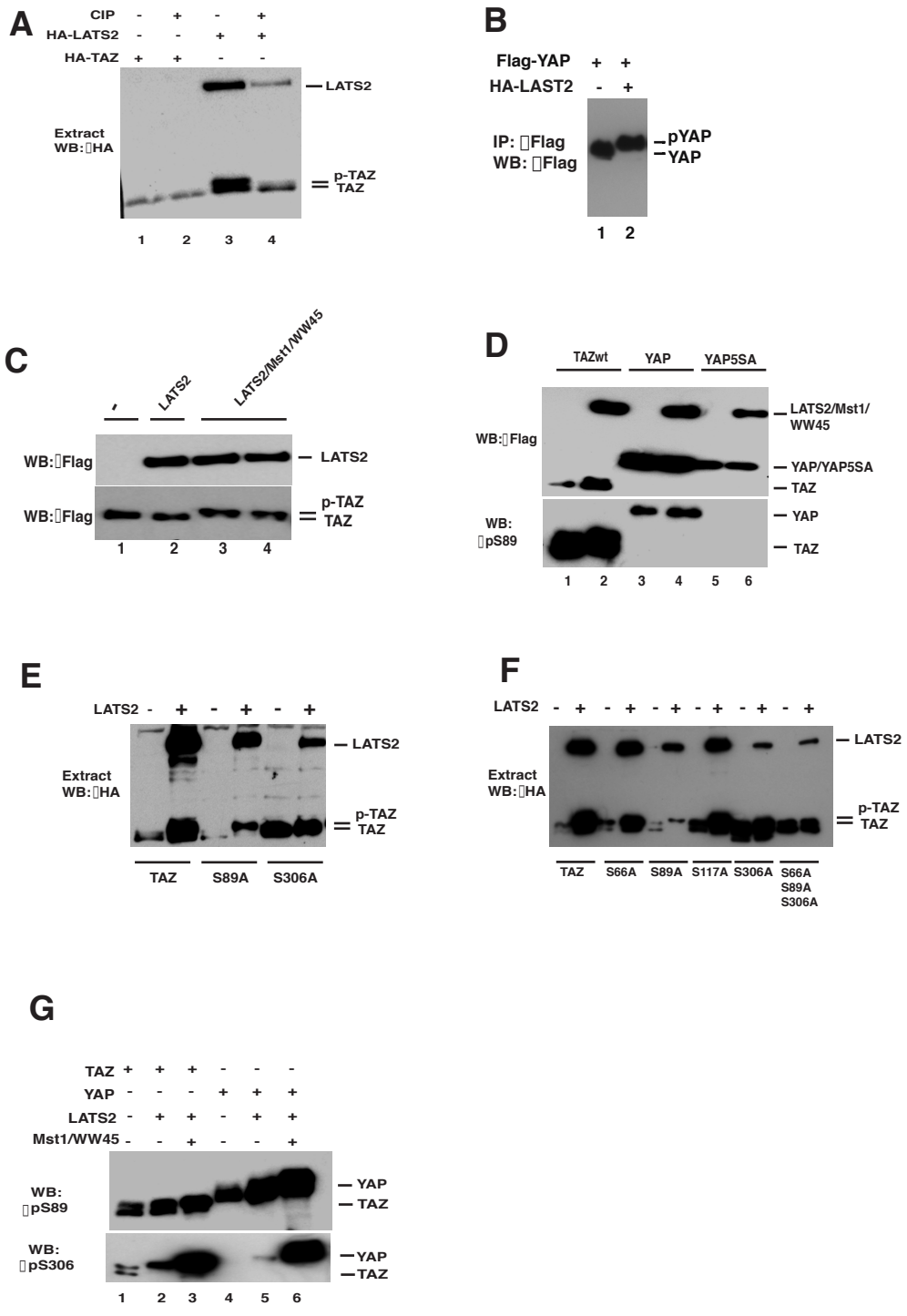

A
CIP - + - +
HA-LATS2 - - + +
HA-TAZ + + - -
LATS2
Extract WB: HA
p-TAZ
TAZ
1 2 3 4
B
Flag-YAP + +
HA-LAST2 - +
IP: Flag
WB: Flag
pYAP
YAP
1 2
C
LATS2
LATS2/Mst1/WW45
WB: Flag
LATS2
WB: Flag
p-TAZ
TAZ
1 2 3 4
D
TAZwt
YAP
YAP5SA
WB: Flag
LATS2/Mst1/ WW45
YAP/YAP5SA
TAZ
WB: pS89
YAP
TAZ
1 2 3 4 5 6
E
LATS2 - + - + - +
Extract WB: HA
LATS2
p-TAZ
TAZ
TAZ
S89A
S306A
F
LATS2 - + - + - + - + - + - +
Extract WB: HA
LATS2
p-TAZ
TAZ
TAZ
S66A
S89A
S117A
S306A
S66A S89A S306A
G
TAZ + + + - - -
YAP - - - + + +
LATS2 - + + - + +
Mst1/WW45 - - + - - +
WB: pS89
YAP
TAZ
WB: pS306
YAP
TAZ
1 2 3 4 5 6

Figure 5

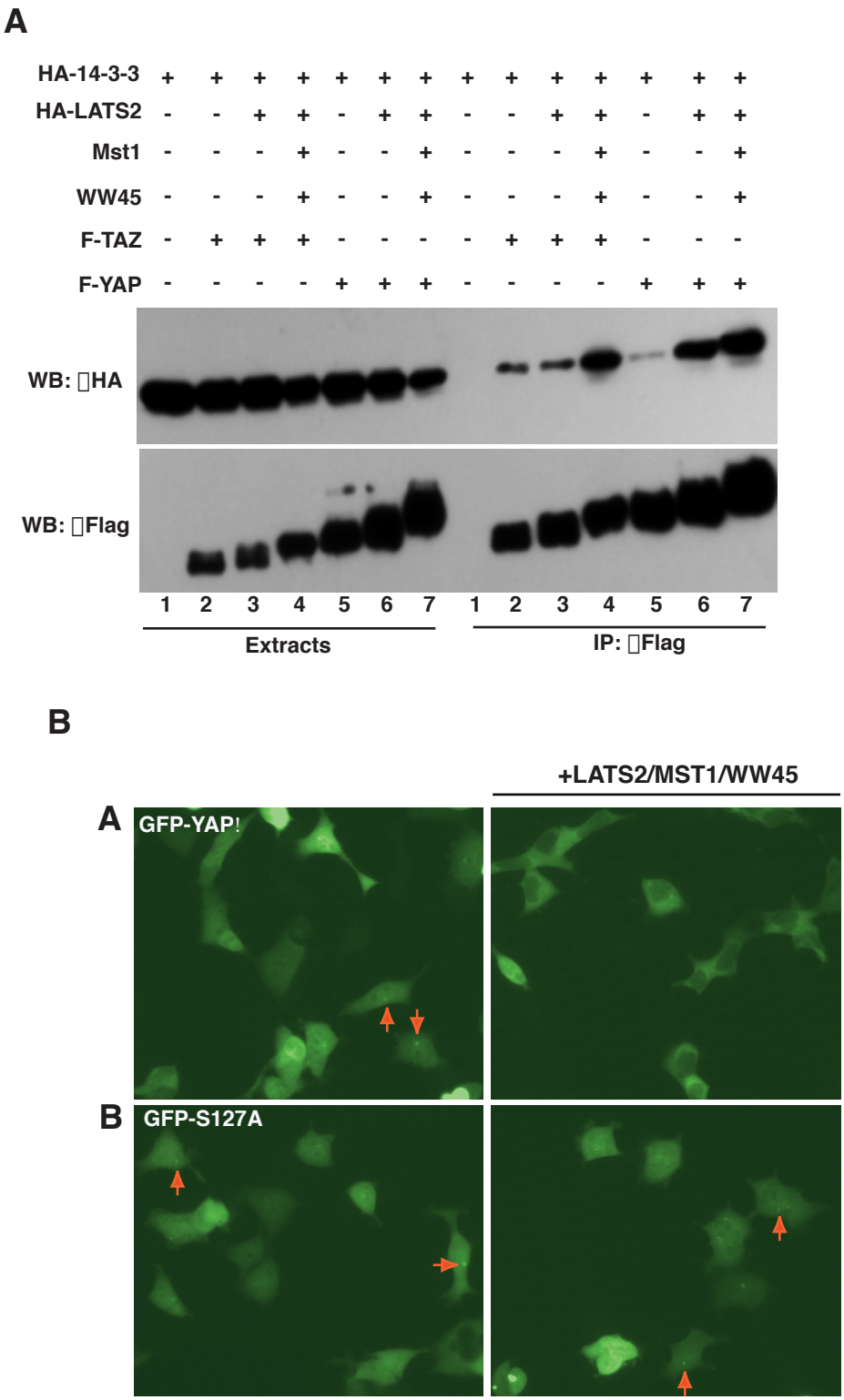

A
HA-14-3-3 + + + + + + + + + + + + + +
HA-LATS2 - - + + - + + - - + + - + +
Mst1 - - - + - - + - - - + - - +
WW45 - - - + - - + - - - + - - +
F-TAZ - + + + - - - - + + + - - -
F-YAP - - - - + + + - - - - + + +
WB: □HA
WB: □Flag
1 2 3 4 5 6 7 1 2 3 4 5 6 7
Extracts
IP: □Flag
B
+LATS2/MST1/WW45
A
GFP-YAP!
B
GFP-S127A

Figure 6

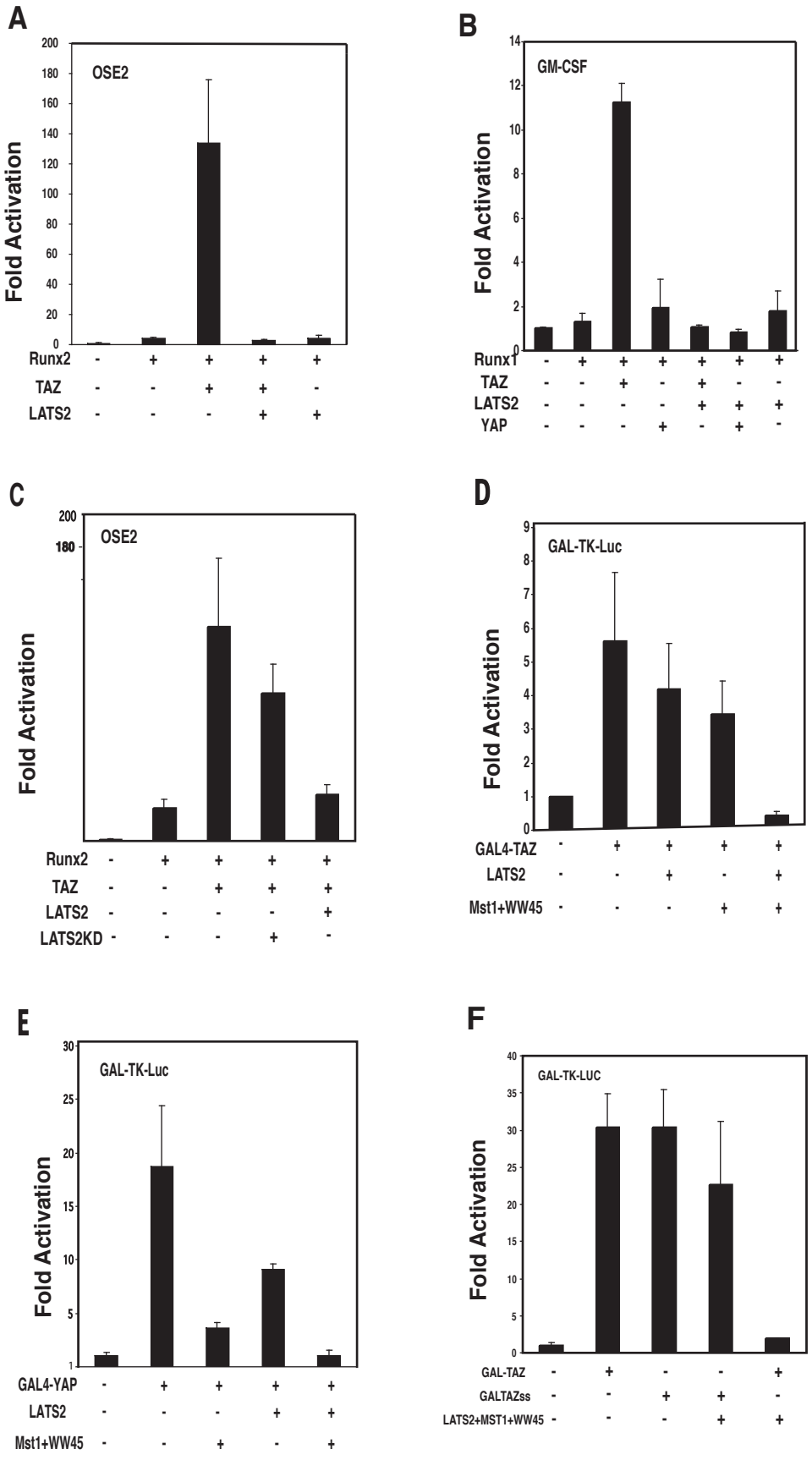

A
OSE2
Fold Activation
200
180
160
140
120
100
80
60
40
20
0
Runx2 - + + + +
TAZ - - + + -
LATS2 - - - + +
B
GM-CSF
Fold Activation
14
12
10
8
6
4
2
0
Runx1 - + + + + + +
TAZ - - + - + - -
LATS2 - - - - + + +
YAP - - - + - + -
C
OSE2
Fold Activation
200
180
Runx2 - + + + +
TAZ - - + + +
LATS2 - - - - +
LATS2KD - - - + -
D
GAL-TK-Luc
Fold Activation
9
8
7
6
5
4
3
2
1
0
GAL4-TAZ - + + + +
LATS2 - - + - +
Mst1+WW45 - - - + +
E
GAL-TK-Luc
Fold Activation
30
25
20
15
10
5
1
GAL4-YAP - + + + +
LATS2 - - - + +
Mst1+WW45 - - + - +
F
GAL-TK-LUC
Fold Activation
40
35
30
25
20
15
10
5
0
GAL-TAZ - + - - +
GALTAZss - - + + -
LATS2+MST1+WW45 - - - + +

Figure 7

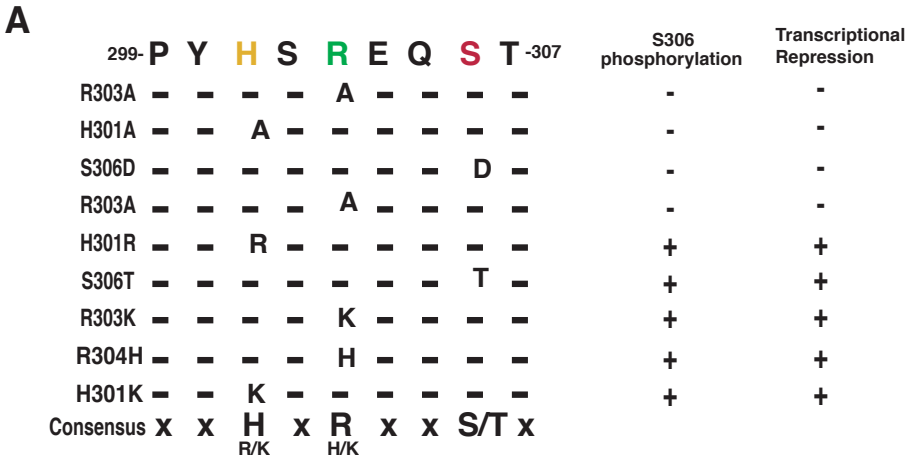

A
299- P Y H S R E Q S T -307
S306 phosphorylation
Transcriptional Repression
R303A A - -
H301A A - -
S306D D - -
R303A A - -
H301R R + +
S306T T + +
R303K K + +
R304H H + +
H301K K + +
Consensus x x H R x x S/T x
R/K
H/K
B
LATS2 + - + - + - + -
— LATS2
Extract
WB: HA
— TAZ/mutants
S306D
H301A
R303A
TAZ

Figure 8

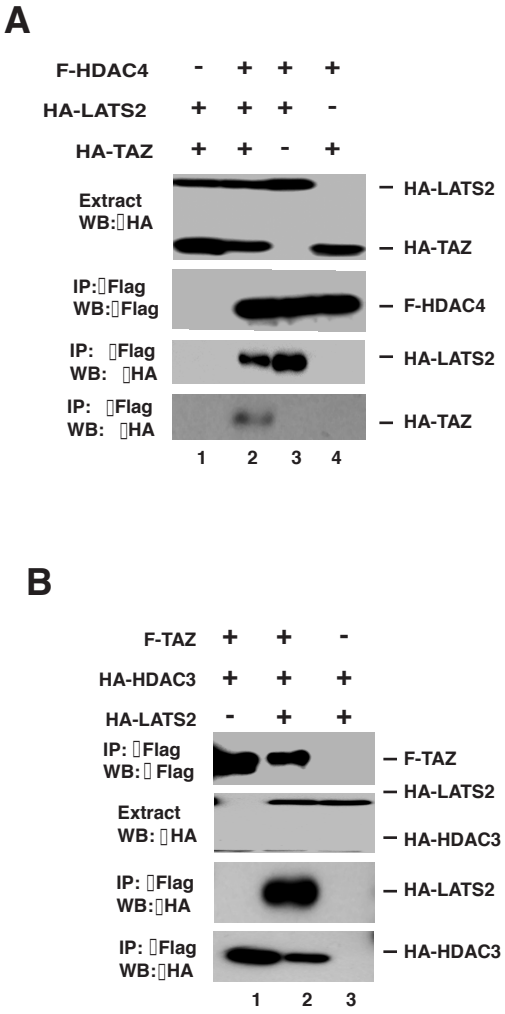
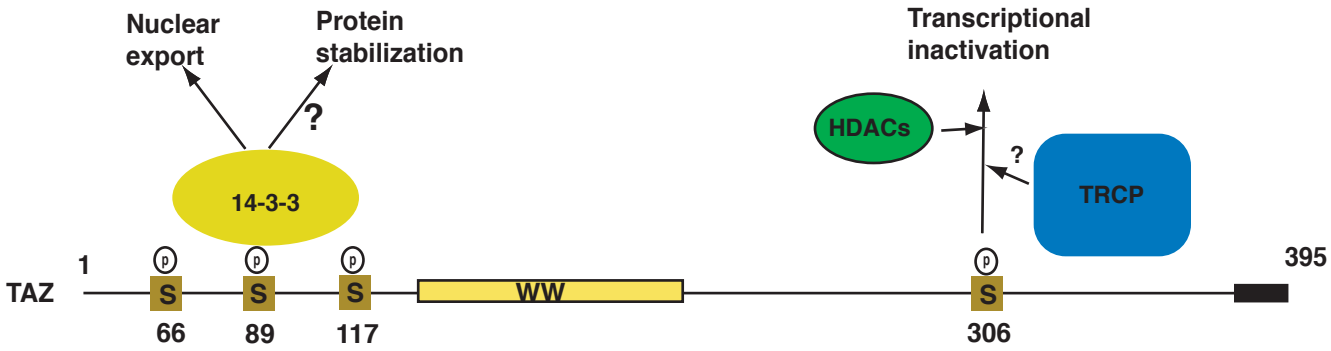

A
F-HDAC4 - + + +
HA-LATS2 + + + -
HA-TAZ + + - +
Extract WB:HA
HA-LATS2
HA-TAZ
IP:Flag WB:Flag
F-HDAC4
IP: Flag WB: HA
HA-LATS2
IP: Flag WB: HA
HA-TAZ
1 2 3 4
B
F-TAZ + + -
HA-HDAC3 + + +
HA-LATS2 - + +
IP: Flag WB: Flag
F-TAZ
HA-LATS2
Extract WB: HA
HA-HDAC3
IP: Flag WB:HA
HA-LATS2
IP: Flag WB:HA
HA-HDAC3
1 2 3

Figure 9


Nuclear
export
Protein
stabilization
?
14-3-3
Transcriptional
inactivation
HDACs
?
TRCP
1
TAZ
P
S
66
P
S
89
P
S
117
WW
P
S
306
395

Chapter 5

Effect of cell-cell contact on the subcellular localization of YAP and TAZ

Minghong Xu, Lin Xiao and Xiang-Jiao Yang

Preface

In Chapter 4, we identified a long sought kinase for TAZ and YAP in mammalian cells. LATS2 is a serine/threonine kinase homologous to Warts/Lats identified in *Drosophila*. We showed that in mammalian cells, LATS2 efficiently phosphorylates TAZ and YAP at several sites. The phosphorylation of TAZ and YAP results in their nuclear export, transcriptional inactivation, and apoptosis-like changes of cells where TAZ or YAP is co-expressed with LATS2, Mst1 and WW45. These results demonstrated the functional consequences of activated *hippo*-like pathway. To achieve full activation of this pathway, the upstream inputs are important. So far, only Merlin/Expanded and Fat have been shown to activate the *hippo*-like pathway, little is known about the initial input. In order to identify such inputs, we performed the studies discussed in this chapter.

Abstract

Previously, we found that TAZ and YAP were the targets of the LATS2 kinase in mammalian cells and demonstrated that phosphorylation of TAZ and YAP by this kinase led to their transcriptional inactivation and nuclear export. Other labs have established that Merlin is activated when cells reach high confluency. Merlin is upstream from the *hippo*-like pathway. To assess whether high confluency could lead to phosphorylation and nuclear export of TAZ and YAP, we examined the effects of cell confluency. Our data showed clearly that YAP and a portion of TAZ could be exported when cells reach high confluency. This effect was independent of the proline-rich domain of YAP. Since binding to 14-3-3 is the major known mechanism by which YAP and TAZ shuttle to the cytoplasm and serine 127 of YAP mediates 14-3-3 binding, we tested several serine mutants containing serine 127 substitution. These results showed that serine 127 was the major site responsible for confluency-mediated nuclear export. Since serine 127 is the 14-3-3 binding site, we hypothesized that when cells were at low confluency, the affinity of YAP for 14-3-3 was low and the binding increased when cells were in close contact. The co-immunoprecipitation data indicated that this was the case. In conclusion, we demonstrated that subcellular localization of YAP and TAZ is regulated by cell-cell contact.

Introduction

One of the most intriguing phenomena in biology is the different organ size among animals. Although significant progress has been made in understanding many signaling pathways in controlling cell proliferation or cell fate determination, little is known regarding how these pathways work together to build up a living organism of a certain size. Organ size is controlled by complex factors including both environmental and biological ones. Largely, the biological factors can be categorized into two main groups: intracellular and extracellular factors (Conlon and Raff, 1999). The intracellular factors are exemplified by the factors that regulate growth stimulatory genes. p27 is one of these. p27 knockout results in 30% increase of mutant animal size and organs from the mutant animals contain more cells (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). The extracellular factors involve signaling pathways that modulate cell growth such as the TGF-β family member, myostatin (McPherron et al., 1997; McPherron and Lee, 1997). Recently, a new emerging pathway, the *hippo* pathway, has been closely linked to the organ size control mechanisms for its ability to restrict cell growth and promote apoptosis (Pan, 2007). The growth control effect of this pathway is achieved by phosphorylation and inactivation of Yorkie, a *Drosophila* orthologue of mammalian YAP.

The intriguing data presented in Chapter 4 revealed detailed mechanisms by which the *hippo* pathway regulated the functions of YAP and its paralogue TAZ in mammalian cells. LATS2 phosphorylates TAZ and YAP at the consensus sites, leading to their transcription inactivation. One of these sites is also responsible for 14-3-3 binding. Association with 14-3-3 results in cytoplasmic retention of TAZ and YAP. It is known

that YAP activates the transcription of cyclin E, Diap1, and Bantam (Nolo et al., 2006; Thompson and Cohen, 2006), all of which positively regulate cell proliferation. Inactivation of transcriptional activity of YAP by phosphorylation and cytoplasmic retention provide detailed mechanisms for the growth inhibitory role of the *hippo*-like pathway.

The upstream elements of this pathway include Merlin and Expanded, members of the ERM (ezrin/radixin/moesin) family. This family of proteins contains the FERM domain and is thought to link transmembrane proteins to the cytoskeleton. Hypophosphorylated Merlin activates *hippo* (Edgar, 2006; Pellock et al., 2006). The phosphorylation state of Merlin is influenced by several factors. Serum starvation is known to activate Merlin (Shaw et al., 1998b). It has been shown that when cultured NIH3T3 cells are not confluent, Merlin is mostly in the hyperphosphorylated form; when the cells become confluent and stop growing, the hypophosphorylated form increases dramatically (Shaw et al., 1998a). The phosphorylation site was identified as serine 518 (Rong et al., 2004).

Growth arrest in cultured monolayer cells is well known to be regulated by some signals that mediate the "contact inhibition". This inhibition has been extensively studied and involves several key players of cell growth control, including p21-activated kinase (PAK), the PAK activator Rac, p27kip1/cyclinD1, p16/INK4, TGFβ (Wieser et al., 1999; Zhang et al., 1999), and the Ca^{2+}-binding protein S100C (Sakaguchi et al., 2000). The *hippo* pathway has also been implicated in regulation of contact inhibition. Besides the aforementioned changes in Merlin phosphorylation induced by confluency, further evidence comes from LATS2 knockout mice (McPherson et al., 2004). LATS2 knockout

is embryonic lethal, but cells derived from this animal showed a tumor-like growth pattern. The cell division continues even when the cells are confluent; Instead of forming a monolayer in the culture dish, the cells grow into foci. This aberrant growth pattern of LATS2$^{-/-}$ cells, together with the finding that Merlin is activated when cells reach confluency, strongly suggest the hippo-like pathway regulates cell growth by sensing cell-cell contact, a key feature of organ size controllers. But how the cellular context signal is translated into a growth stop sign is not fully understood. In light of the findings that ectopic expression of LATS2 results in nuclear export of TAZ and YAP (Chapter 4), we considered and investigated whether and how cell-cell contact might exert its effect in a similar fashion and serve as a brake for cell proliferation.

Materials and methods

Cell culture — Human embryonic kidney (HEK293) cells and mouse NIH3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin and streptomycin (Invitrogen).

Mutagenesis of YAP mutants — Long-fidelity based PCR (Roche) was used to generate YAP mutants as described in chapter 4.

Live green fluorescence microscopy — GFP-tagged YAP wild type and mutants were transfected into HEK293 or NIH3T3 cells by Superfect (Qiagen). 24 h later, GFP signals were observed by fluorescence microscopy. After that, cells in some wells were allowed to grow for additional 48 h to reach confluency. Pictures were taken and processed using Photoshop.

Co-immunoprecipitation and Western blot — HEK293 cells were transfected with the expression plasmids using Superfect (Qiagen). 48 h after transfection, cells were harvested in buffer B150 (20 mM Tris-HCl [pH 8.0], 10% glycerol, 0.15 M KCl, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) plus 50 mM NaF. Immunoprecipitation was performed by incubation of M2 agarose beads (Sigma) with cell lysates for 2 h at 4°C. The beads were washed 4 times with the same buffer. Bound proteins were eluted with Flag peptide (Sigma). Eluted proteins were mixed with SDS sample buffer and resolved by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane followed by Western blotting as described previously (Chapter 2).

Results

Subcellular localization of TAZ and YAP is regulated by cell-cell contact

Although it is unclear about the initial inputs that trigger the activation of the hippo-like pathway in mammalian cells, cell-cell contact has been implicated in Merlin activation (Shaw et al., 1998a; Shaw et al., 1998b). Our previous studies have demonstrated that activation of the *hippo*-like pathway in mammalian cells caused nuclear export of TAZ and YAP (Chapter 4). To link cell-cell contact to nuclear-cytoplasmic trafficking of TAZ and YAP, we transfected GFP-tagged YAP and TAZ into HEK293 cells at either 40% or 100% confluency. The results showed that when the cells were at low confluency, YAP was mostly pancellular (Fig. 1A). When the cells reached confluency, YAP became predominately cytoplasmic (Fig. 1A). Clearly, YAP showed confluency-dependent subcellular localization. Similarly, YAP was mostly pancellular in cultured cells on day one when cells were about 40% confluent. On day three after transfection, there was a drastic change of the localization of YAP (Fig. 2C). TAZ showed similar pattern but with less efficiency (Fig. 1B and see text below). In conclusion, cell-cell contact is a sufficient stimulus for nuclear export of TAZ and YAP.

Confluency-induced nuclear export of YAP is cell line-dependent

To test if this phenomenon is specific to HEK293 cells, we repeated the same experiment in NIH3T3 fibroblast (Fig. 2A). At low confluency, there were two major populations of cells in regard to YAP subcellular distribution: cells containing YAP in the cytoplasm (data not shown) and those with pancellular YAP distribution (Fig. 2A). In response to increased confluency, YAP became cytoplasmic (data not shown) even in highly GFP-

saturated cells (Fig. 2A, arrow head). The localization of YAP contrasts between HEK293 and NIH3T3 cells. This could be due to the difference of the endogenous kinase activity. Indeed, by Western blotting, we detected more hyperphophorylated YAP in NIH3T3 than HEK293 cells, especially when WW45 and Mst1 were co-expressed with LATS2 (data not shown). Taken together, these results indicate that the *hippo*-like pathway is more active in NIH3T3 cells, thereby leading to more efficient nuclear export of TAZ and YAP.

We also tested ROS17/2.8 cells, a rat osteosarcoma cell line, HeLa and MDCK cells. We did not observe obvious nuclear export of YAP in response to cell-cell contact in these cells (data not shown). For example, in HeLa cells, YAP was predominantly cytoplasmic regardless of the confluency (data not shown). In conclusion, our results demonstrate that the subcellular localization of YAP is regulated by cell-cell contact in a cell line-dependent manner.

The proline-rich domain of YAP is not important for nuclear export

We performed similar experiments on TAZ along with YAP. The results showed that TAZ also responded to cell confluency changes but to a lesser extent (Fig. 1B). In 30% of the cells, TAZ did not shuttle in response to cell-cell contact (data not shown). TAZ and YAP are highly homologous to each other (Chapter 4). Similarity in primary structure suggests similar functions. But the disparate pattern of nuclear shuttling between TAZ and YAP in response to cell-cell contact suggests a distinct nature of TAZ. Indeed, sequence comparison revealed a major difference between TAZ and YAP. TAZ lacks the N-terminal proline-rich domain present in YAP. We asked if this domain was important

in mediating confluency-induced nuclear export. The results showed that when the cells were at low density, YAPΔ, which lacks the proline-rich domain, tended to be more enriched in the nucleus than wild type YAP (data not shown). But when cells were at high density, YAPΔ was still exported into the cytoplasm (Fig. 1C). Consistent with this, co-immunoprecipitation experiments did not show significant difference between YAP and YAPΔ in binding to 14-3-3 (Fig. 3B). We concluded that the proline-rich domain played a minimal role in the nuclear-cytoplasmic shuttling of YAP. The structure that confers the disparity between TAZ and YAP remains to be determined.

Serine 127 mediates cell-cell contact-induced nuclear export of YAP

It is known that binding to 14-3-3 regulates the subcellular localization of YAP. The site for 14-3-3 binding is serine 127. We reasoned that this serine might be responsible for the nuclear export of YAP in response to cell confluency. To examine this, we first set out to test if serine 127 is phosphorylated in response to cell confluency. For this, we utilized anti-pS89 antibody. It also detects phospho-serine 127 in YAP. Western blot analysis showed that YAP, when expressed in cells at low density, exhibited a basal level of phosphorylation of serine 127. But when cells were confluent, a significant increase in phosphorylation of this serine was detected (Fig. 3A). Consistent with this finding, 14-3-3 binding to YAP was significantly increased in response to cell-cell contact (Fig 3C). These results suggest that serine 127 phosphorylation mediates the nuclear export of YAP. Indeed, the YAP S127A mutant responded much less efficiently to the confluence changes in regard to nuclear export (Fig. 4A, 4B).

Our previous studies identified several other sites in TAZ and YAP phosphorylated by LATS2. We asked if other sites could also contribute to the nuclear export of YAP. Our data showed that the mutations of serines 109 and 347 did not affect the localization of YAP (Fig. 4 and data not shown). Taken together, these results demonstrate that confluency-mediated serine 127 phosphorylation and subsequent 14-3-3 binding comprise major mechanisms controlling the subcellular localization of YAP, and perhaps TAZ, in response to cell-cell contact.

Discussion

Our findings in Chapter 4 demonstrate that TAZ and YAP are phosphorylated by LATS2, a serine/threonine kinase. But the initial input that triggers activation of LATS2 itself was unknown. In this chapter, we uncovered cell-cell contact as a sufficient stimulus to activate LATS2, manifested as cell confluency-dependent subcellular localization of YAP. This confluency-induced subcellular localization change is conferred by serine 127 of YAP. Serine 127 mutant showed pancellular localization while wild type YAP is predominately cytoplasmic when cells are confluent. Consistent with this result, we showed that the affinity between14-3-3 and YAP is also confluency-dependent. The affinity is higher when cells are in close contact.

Regulation of YAP by the hippo-like pathway

YAP is important for mammalian development. Knockout mice died early in embryonic development (Morin-Kensicki et al., 2006). The embryo showed defects in yolk sac vasculogenesis, chorioallantoic attachment, and embryonic axis elongation. Although no direct cell-based study confirms the growth stimulatory role of YAP in mammalian cells, the studies in *Drosophila* suggest that YAP positively regulates growth (Huang et al., 2005) and controls organ size (Dong et al., 2007). Some evidence even suggests that YAP is an oncogene (Zender et al., 2006). This study scanned the genome of human hepatocellular carcinoma and found that YAP is in an amplicon of 11q22. This suggests overexpression of YAP is involved in the tumorigenesis. Indeed, prolonged expression of YAP in mouse liver leads to carcinoma formation (Dong et al., 2007). In addition, a group reported YAP is involved in controlling epithelium-mesenchyme-trasition (EMT) (Overholtzer et al., 2006), a crucial step in tumor development and metastasis. These

results strongly suggest that YAP is an important regulator of growth in a physiological state, and its deregulation causes cancer.

But little is known about the regulation of YAP. An important line of research is identifying the kinases that regulate the function of TAZ and YAP. Akt has been shown to phosphorylate YAP at serine 127, which leads to cytoplasmic retention and decreased transcription co-activator activity. But this cannot be reproduced in our laboratory (Goh, S.L. & Yang, X.J., unpublished data). The study on Yorkie in *Drosophila* brought up the possibility that mammalian YAP might be under control of the *hippo* pathway (Huang et al., 2005). Consistent with this, our previous study demonstrate that LATS2 is a potent kinase for TAZ and YAP in mammalian cells (Chapter 4). Phosphorylation of YAP and TAZ by LATS2 leads to their transcriptional inactivation and nuclear export. The hippo-like pathway is known to be activated by high density of cultured cells. In cultured NIH3T3 cells, Merlin, an upstream input of the *hippo* pathway, is activated when the cells are confluent (Shaw et al., 1998b), both hyper- and hypophosphorylated Merlin accumulate in the cells. The hypophosphorylated form activates the *hippo* pathway through an unknown mechanism. Our study clearly linked all these findings by proving that cell-cell contact induces activation of endogenous *hippo* pathway which in turn phosphorylates YAP and TAZ, thereby resulting in their nuclear export.

14-3-3 is important in the regulation of YAP function

It is known that serine 127 is the site for 14-3-3 binding and one of the sites phosphorylated by the hippo-like pathway (Chapter 4), this prompted us to test the role of 14-3-3 binding in the confluency-induced subcellular trafficking of TAZ and YAP. First,

we sought to test whether YAP S127A mutant could behave differently if binding to 14-3-3 is the main mechanism by which YAP is exported out of the nucleus. Our finding confirmed that serine 127 was one of the main players in YAP nuclear export since YAP S127A failed to export in response to cell confluency.

Our results suggest that confluency-induced nuclear export of TAZ and YAP is cell line specific as in MDCK, HeLa and ROS17/2.8 cells, no such observation was made (data not shown). Of particular note is the phenomenon that we observed in NIH3T3 cells at low density. The distribution of transfected YAP is divided into mainly two populations: pancellular and cytoplasmic. The nuclear export of those with pancellular distribution is dramatic even in highly GFP signal-saturated cells. Consistent with this, Western blot showed a more efficient phosphorylation of YAP. These findings point to a more active hippo-like pathway in NIH3T3 cells.

Future directions

In the future, siRNA experiments will be needed to further confirm that nuclear export of YAP and TAZ is through LATS2 kinase. Mass spectrometry will also be valuable to identify more phosphorylated serines in TAZ or YAP. Once the serines are identified, we can monitor the experimental conditions to get more physiological relevant factors controlling subcellular localization of TAZ and YAP. Another important aspect is to generate more phosphoserine specific antibodies, and use these antibodies to study endogenous TAZ and YAP. All of these experiments will greatly benefit our understanding of the *hippo*-like pathway and the properties of YAP and TAZ, thereby

shedding light on some fundamental issues related to mammalian organ size control and tumorigenesis.

References

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

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*, 1120-1133.

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

Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak, K., Tsai, L. H., Broudy, V., Perlmutter, R. M.*, et al.* (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell *85*, 733-744.

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*, 421-434.

Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A., and Koff, A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell *85*, 721-732.

McPherron, A. C., Lawler, A. M., and Lee, S. J. (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature *387*, 83-90.

McPherron, A. C., and Lee, S. J. (1997). Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci U S A *94*, 12457-12461.

McPherson, J. P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak-Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J., *et al.* (2004). Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. Embo J *23*, 3677-3688.

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*, 77-87.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., and Loh, D. Y. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell *85*, 707-720.

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*, 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*, 12405-12410.

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

Pellock, B. J., Buff, E., White, K., and Hariharan, I. K. (2006). The Drosophila tumor suppressors Expanded and Merlin differentially regulate cell cycle exit, apoptosis, and Wingless signaling. Dev Biol.

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*, 8447-8454.

Sakaguchi, M., Miyazaki, M., Inoue, Y., Tsuji, T., Kouchi, H., Tanaka, T., Yamada, H., and Namba, M. (2000). Relationship between contact inhibition and intranuclear S100C of normal human fibroblasts. J Cell Biol *149*, 1193-1206.

Shaw, R. J., McClatchey, A. I., and Jacks, T. (1998a). Localization and functional domains of the neurofibromatosis type II tumor suppressor, merlin. Cell Growth Differ *9*, 287-296.

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

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*, 767-774.

Wieser, R. J., Faust, D., Dietrich, C., and Oesch, F. (1999). p16INK4 mediates contact-inhibition of growth. Oncogene *18*, 277-281.

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., *et al.* (2006). Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. Cell *125*, 1253-1267.

Zhang, H. S., Postigo, A. A., and Dean, D. C. (1999). Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. Cell *97*, 53-61.

Figure 1

The subcellular localization of YAP and TAZ is regulated by cell-cell contact

(A) HEK293 cells, seeded at both low and high density, were transfected with expression plasmids for GFP-YAP protein. GFP signals were analyzed by live fluorescence microscopy 24 h after transfection. In cells with low density, YAP is predominately pancellular. In cells with high confluency, YAP is mostly cytoplasmic (bottom panel).

(B) Expression plasmids for GFP-TAZ protein were transfected into HEK293 cells seeded at both low and high density. 24 h after transfection, GFP signals were analyzed by live fluorescence microscopy. In cells with low density, the distribution of TAZ is divided into two types. One type is pancellular, TAZ with this distribution pattern shuttles out of the nucleus when cells are confluent (bottom panel). The other type is pancellular with nuclear enrichment. The distribution of TAZ in response to cell-cell contact remains unchanged in this type.

(C) For GFP-YAPΔ (a YAP mutant that lacks the N-terminal proline-rich domain), experiments were performed as described in panel A of this figure. GFP-YAPΔ shuttles between the nucleus and cytoplasm in a similar fashion to wild type YAP.

Figure 2

Cell line-dependent subcellular localization of YAP in response to cell-cell contact

(A) NIH3T3 cells, seeded at both low and high density, were transfected with expression plasmids for GFP-YAP protein. GFP signals were analyzed by live fluorescence microscopy 24 h after transfection. In cells with low confluency, YAP is mostly pancelluar or cytoplasmic (not shown). High density of cells results in cytoplasmic translocation of YAP, even in highly GFP signal-saturated cells (arrow head).

(B) Nuclear export of YAP in HEK293 cells. Experiments were performed as descried in panel A. In HEK293 cells, YAP is pancellular. High density leads to efficient nuclear export of YAP.

(C) To discern the dynamic changes of the subcellular localization of YAP in response to confluency, HEK293 cells at low density were transfected with expression plasmid for GFP-YAP protein. Cells were allowed to grow for 3 days and to reach confluency. Note the confluency-induced nuclear export of wild type YAP in HEK293 cells.

Figure 3

The binding of 14-3-3 to YAP is cell confluency-dependent

(A) Cell confluency-dependent phosphorylation of serine 127 in YAP. HEK293 cells at different confluency were transfected with expression plasmid for F-YAP protein. 24 h later, cells were harvested in buffer B150 (20 mM Tris-HCl [pH 8.0], 10% glycerol, 0.15 M KCl, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) plus 50 mM NaF. Both anti-pS89 and anti-flag antibodies were used to detect YAP. When cells are confluent, there is a significant increase in serine 127 phosphorylation of YAP.

(B) HEK293 cells at high density were transfected with expression plasmids for YAP, YAPΔ, YAPS127A and 14-3-3 proteins as indicated. After 48 h, co-immunoprecipitation was performed by incubation of M2 agarose beads with cell lysates for 2 h at 4°C. The beads were washed 4 times with the same buffer. Bound proteins were eluted with Flag peptide. Eluted proteins were mixed with the SDS sample buffer and resolved in SDS-PAGE. The proteins were transferred onto nitrocellulose membrane for Western blotting as described previously (Chapter 2). Both YAP and YAPΔ bind to 14-3-3 when cells are in high density. As expected, the YAP S127A does not bind to 14-3-3.

(C) HEK293 cells, seeded in both high and low density were transfected with expression plasmids for F-YAP, F-YAPΔ and HA-14-3-3 proteins as indicated. After 48 h, co-immunoprecipitation was performed as described in panel B. Both YAP and YAPΔ bind to 14-3-3 when cells are at high density. The binding is very weak when the cells are in low confluency.

Figure 4

Time-point study on nuclear export of YAP and its mutants

(A) Expression plasmids for GFP-YAP wild type and mutant YAP proteins were transfected into HEK293 cells. GFP signals were analyzed on day 1 when the cells were in low density as shown by the phase contrast images on the right side. The YAP S127A, YAP S347A and YAP 5SA (mutation of serines 61, 109, 127, 164, 347 into alanine) are pancellular distribution.

(B) Expression plasmids for wild type and mutant YAP proteins were transfected into HEK293 cells. GFP signals were analyzed on day 3 when the cells are in high density. YAP S127A and YAP 5SA fail to translocate into the cytoplasm in response to cell-cell contact. The YAP S347A behaves in a similar fashion to the wild type YAP.

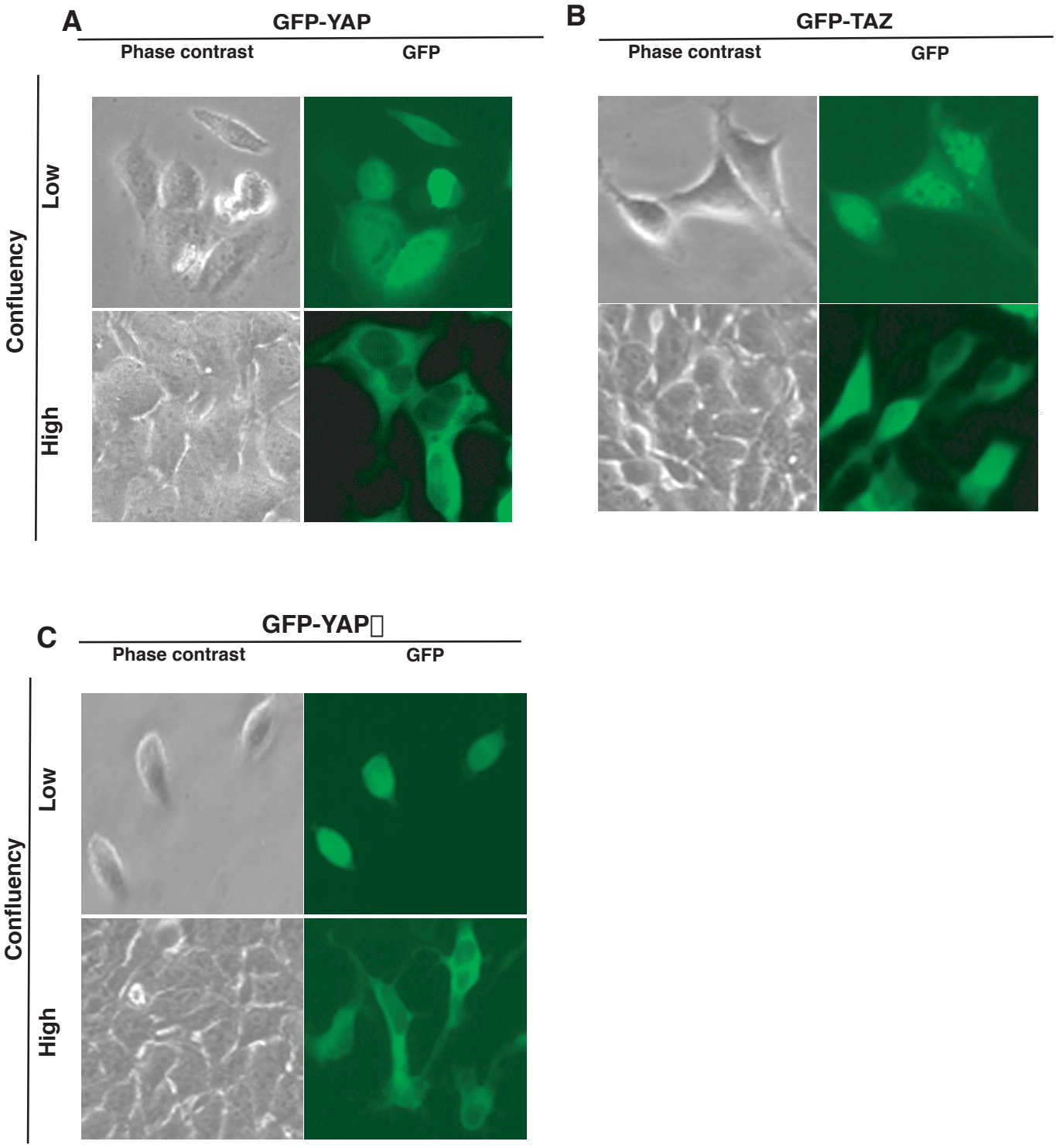
Figure 5

Model illustrating that cell-cell contact results in the activation of *hippo*-like pathway and nuclear export of YAP

(A) Cartoon shows that when cells are not confluent, YAP is predominately pancellular. Nuclear portion of YAP activates transcription of genes such as Bantam, Diap1 and cyclin E.

(B) When cells are confluent, the hippo-like pathway is activated by cell-cell contact. The activation of LATS2 leads to phosphorylation of serine 127 in YAP. Phospho-YAP binds to 14-3-3 strongly, leading to cytoplasmic retention. Activated LATS2 also phosphorylates serine 347 in YAP leading to direct transcriptional inactivation. Both cytoplasmic retention and direct transcriptional inactivation lead to a stop of downstream gene transcription by YAP.

Figure 1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GFP-YAP
Phase contrast
GFP
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Low
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B
GFP-TAZ
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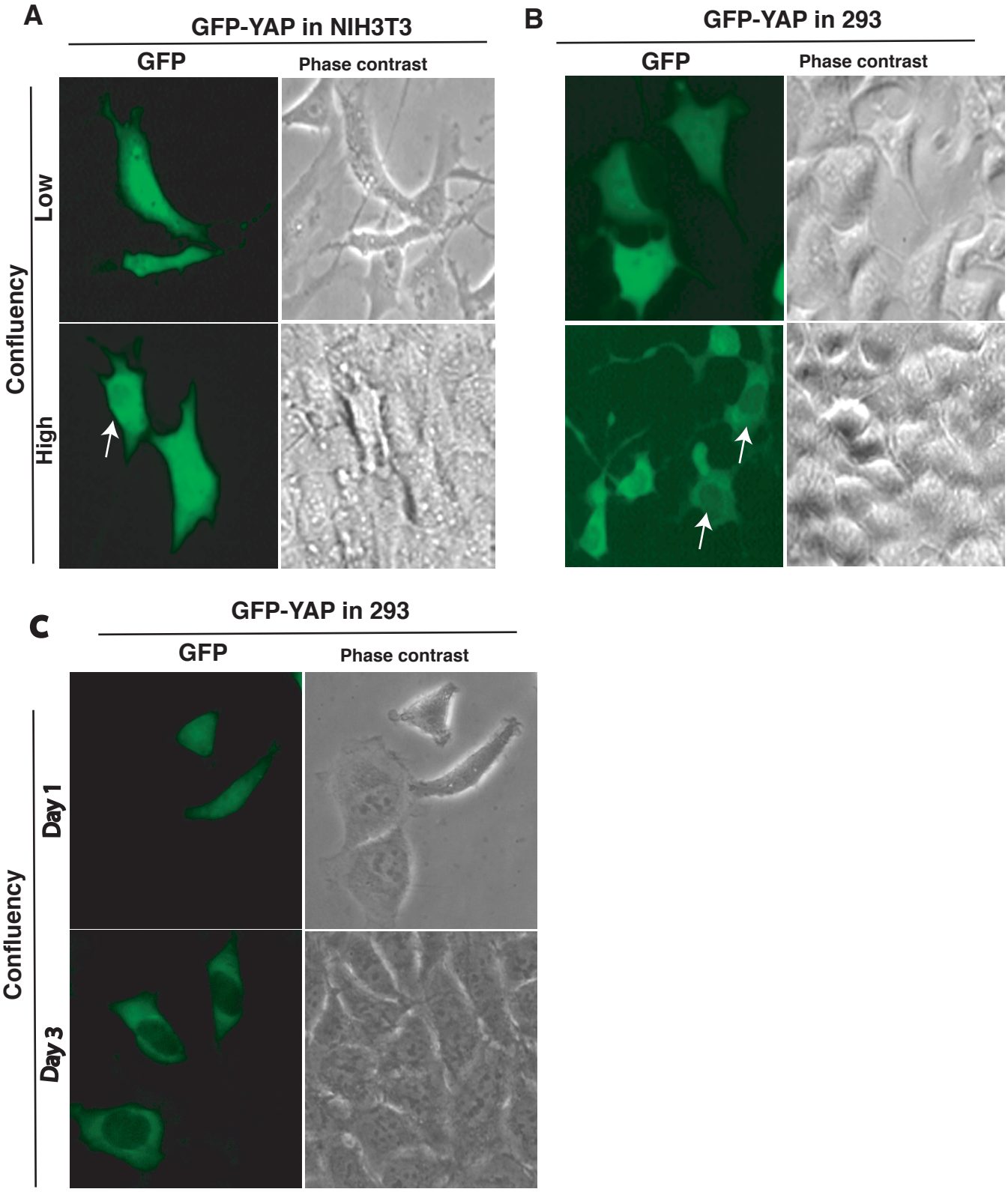
Phase contrast
GFP
Confluency
Low
High

Figure 2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GFP-YAP in NIH3T3
GFP
Phase contrast
Confluency
Low
High
B
GFP-YAP in 293
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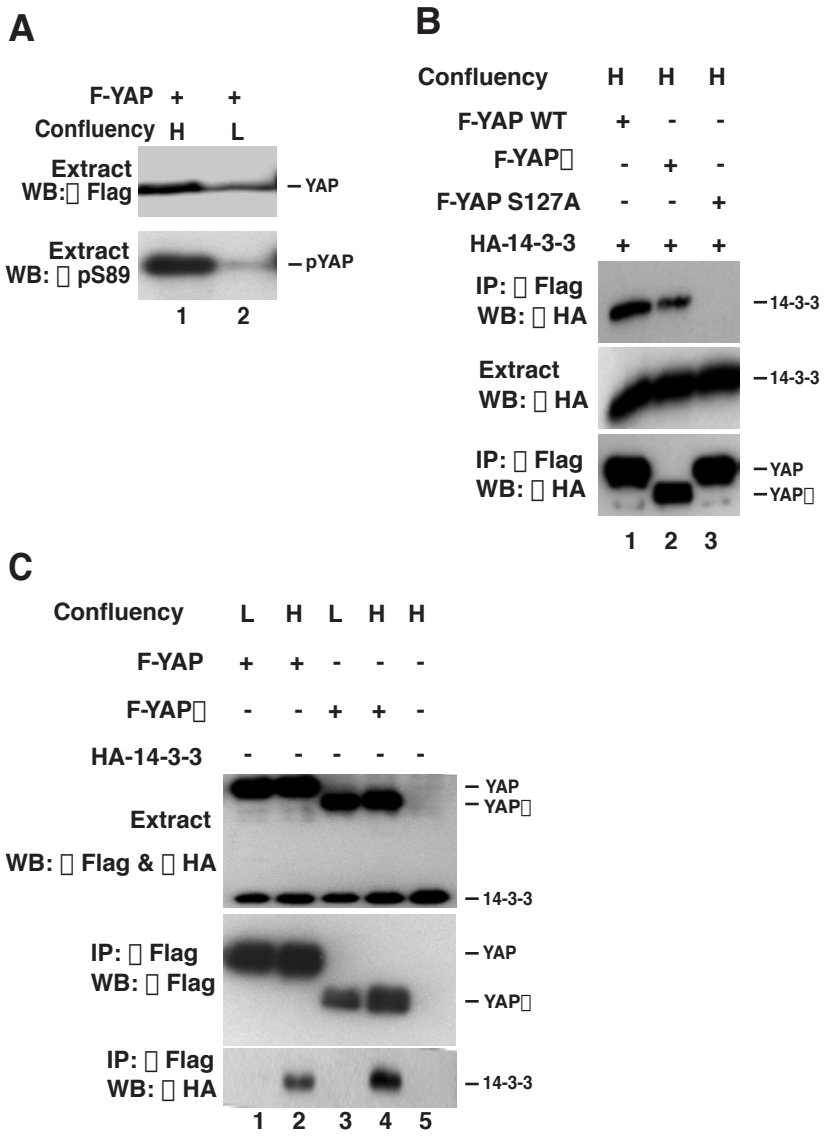
Phase contrast
C
GFP-YAP in 293
GFP
Phase contrast
Confluency
Day 1
Day 3

Figure 3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Confluency H L
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YAP
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1 2
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Confluency H H H
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F-YAP - + -
F-YAP S127A - - +
HA-14-3-3 + + +
IP: Flag WB: HA
14-3-3
Extract WB: HA
14-3-3
IP: Flag WB: HA
YAP
YAP
1 2 3
C
Confluency L H L H H
F-YAP + + - - -
F-YAP - - + + -
HA-14-3-3 - - - - -
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Figure 4

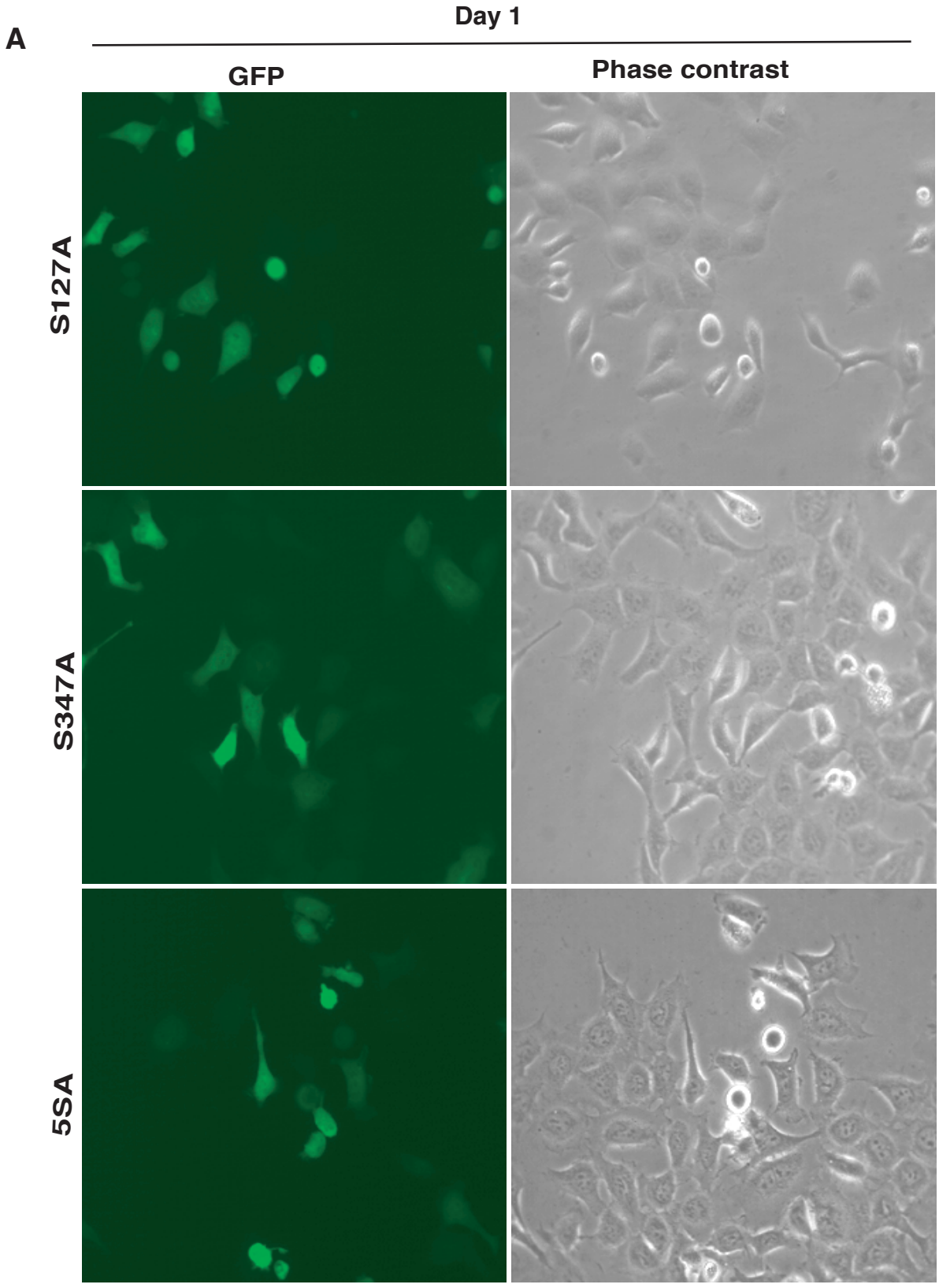


Figure 4

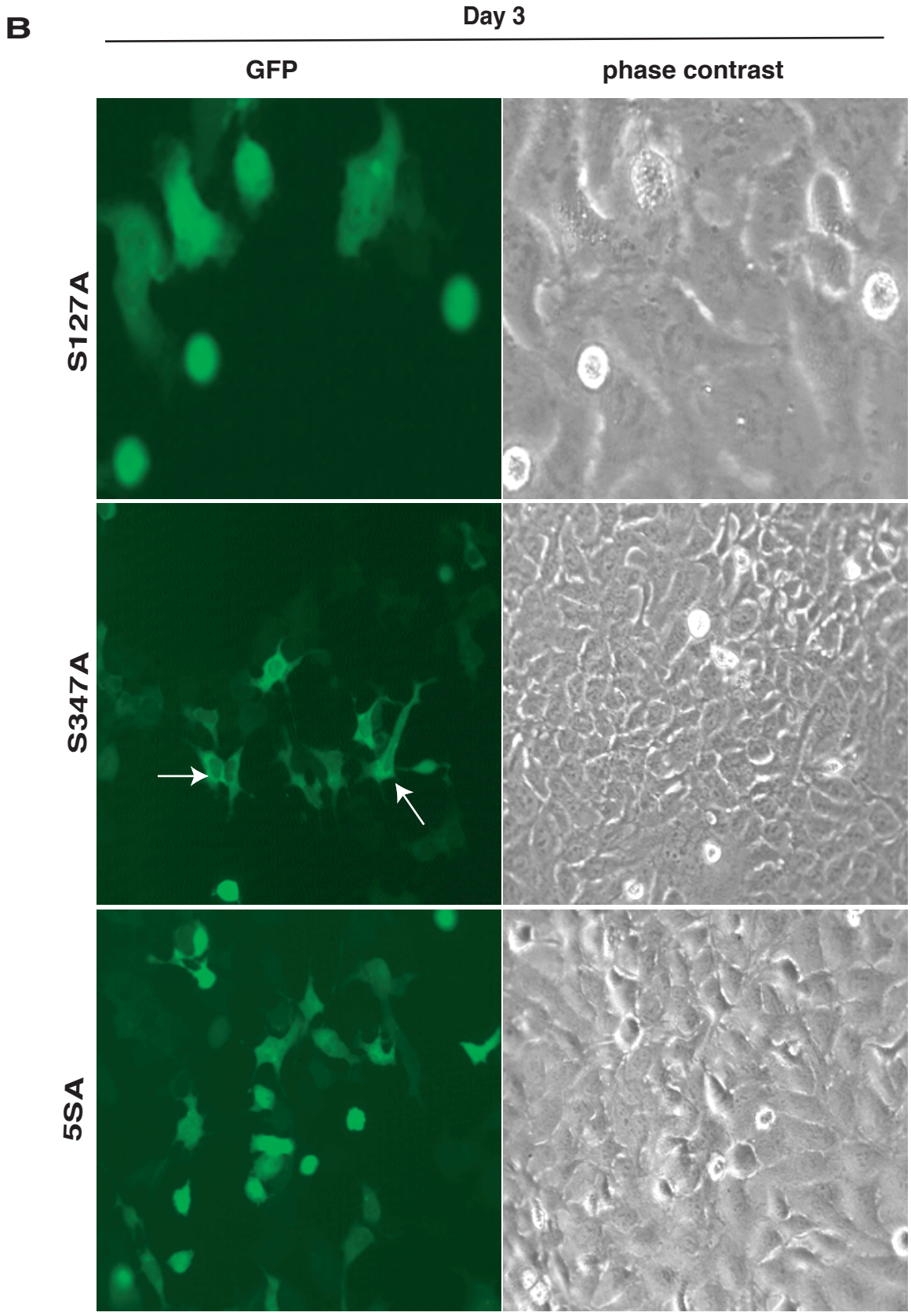
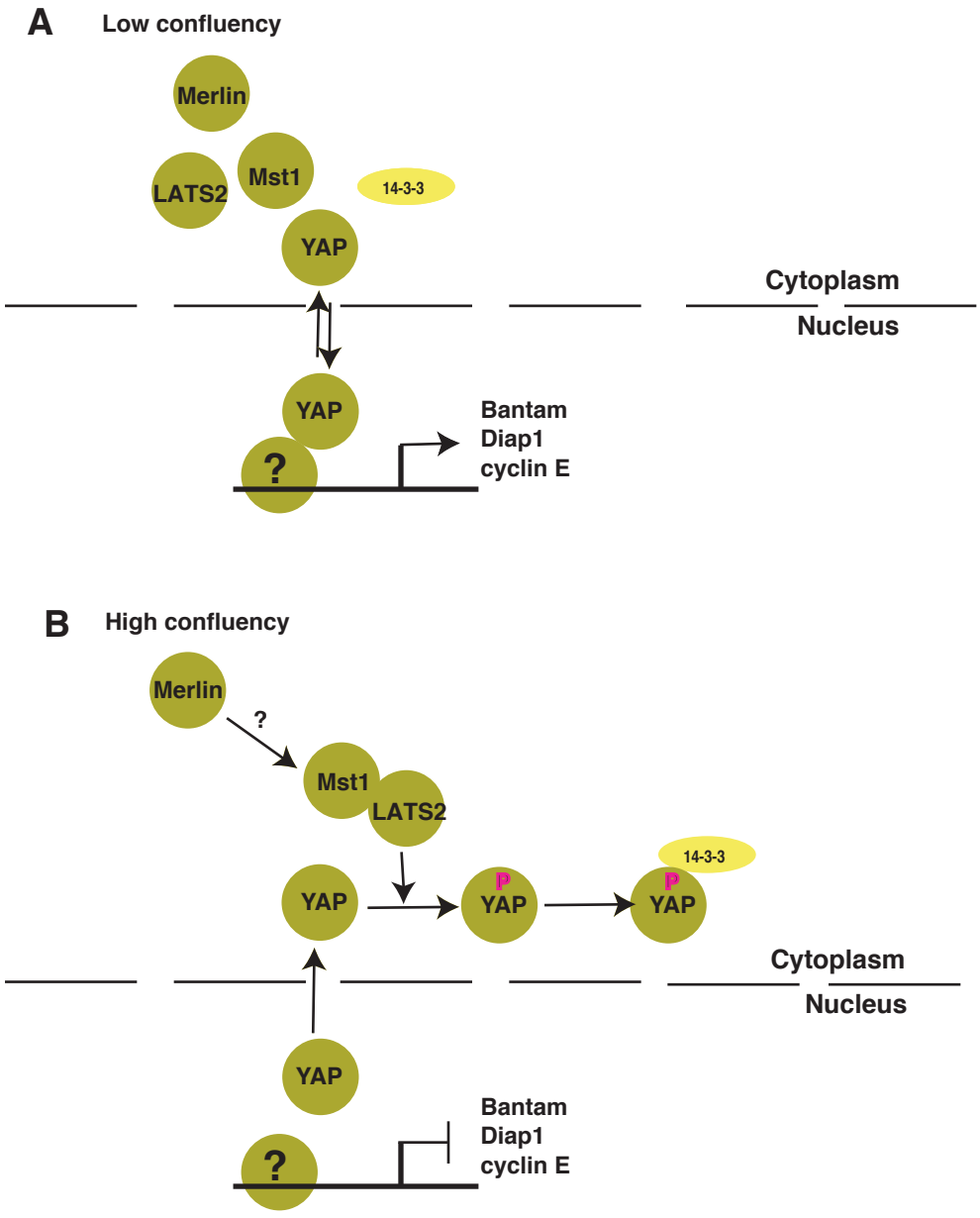


Figure 5


A
Low confluency
Merlin
LATS2
Mst1
14-3-3
YAP
Cytoplasm
Nucleus
YAP
?
Bantam
Diap1
cyclin E
B
High confluency
Merlin
?
Mst1
LATS2
14-3-3
YAP
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YAP
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YAP
Cytoplasm
Nucleus
YAP
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Bantam
Diap1
cyclin E

Chapter 6

General Discussion

In the past 50 years, the transcription field has evolved tremendously. The emergence of the concept of transcription activator was ground breaking and marked the start of the modern transcription field. Soon afterwards, researchers found out the mechanism that an activator binds to DNA and recruits the basal transcription machinery to activate transcription, thereby having a picture of how an activator works. After nearly two decades, another surge in the transcription field came when researchers were dealing with nuclear receptor activators in the early 1990s (Barettino et al., 1994; Danielian et al., 1992; Durand et al., 1994; Glass et al., 1997). From then, a more detailed picture of how an activator works came into being: some activators activate transcription through co-factors while others directly communicate with RNA Pol II.

The discovery of chromatin modification enzymes in the mid 1990s and the proposal of the histone code (Jenuwein and Allis, 2001) added the epigenetic regulations to transcription, and depicted gene transcription as a complex bio-reaction involving many protein complexes and may even involve three dimensional nuclear structure modification and cell cycle progression (Chakalova et al., 2005). Now, the view of activation of a gene is not simply DNA binding and mRNA synthesis, but a complicated matter of when, where and how. In other words, in order for a gene to be transcribed, it awaits signals (when) and subsequent epigenetic modification (how) at certain nuclear sub-domains (where) (Harrington et al., 2002; Stein et al., 2003; Zaidi et al., 2004).

6.1 Mechanisms of action of co-factors for transcription factor — co-activation

In Chapter 1, I reviewed some of the mechanisms, amongst many others, of how co-factors activate gene transcription and how signaling pathways facilitate this process. Previously, MOZ and MORF have been shown to be the co-activators of Runx family

transcription factors (Pelletier et al., 2002). Since TAZ and YAP also function as co-activators of Runx proteins, we investigated how different co-factors work together to activate Runx-dependent transcription. We showed that the co-activators synergize to activate gene transcription (Fig. 1, Chapter 2). The synergistic phenomenon is often seen in enzymatic reactions. In a reaction that is catalyzed by more than one enzyme, one enzyme lowers the threshold to facilitate the rest of the enzymes, making the reaction proceed exponentially. In gene transcription, similar scenarios are seen. Based on the properties of MOZ and TAZ, I propose a model involving the different properties of MOZ and TAZ. MOZ is a histone acetyltransferase (Borrow et al., 1996; Hilfiker et al., 1997), thus it has the potential to modify chromatin structures; while TAZ lacks this function and is thought to contact the basal transcription machinery and activate transcription (Hong et al., 2005). The model is that when both MOZ and TAZ are present, MOZ could significantly decrease the transcription resistance and TAZ would exert its effect fully.

Secondly and more importantly, we showed that TAZ and MOZ formed a tightened co-activator complex upon signal stimulation, which, in my opinion, forms the molecular basis for a gene to be activated or upregulated (Fig. 4, Chapter 2). In the ELISA experiment, while we only observed a mild increase in Runx2-dependent transcription and TAZ-mediated co-activation without PMA treatment, a significant increase was observed for both Runx2-dependent transcription and TAZ mediated co-activation in the presence of PMA. I think this is due to, at least in part, PMA-mediated stronger affinity between proteins although we cannot rule out a PMA-dependent chromatin modification event. Deciphering the mechanisms of how co-factors like TAZ

and MOZ work adds further important insights to the regulation of Runx-dependent transcription that carries extremely important physiological and pathological functions. Deregulation of Runx proteins have been implicated in many types of cancer (Ito et al., 2003), and Runx1 has been referred to as the most mutated transcription factor in leukemia (Ito, 2004).

6.2 Mechanisms of action of co-factors for transcription factor — co-repression

TAZ, under certain circumstances, can also be a co-repressor (Hong et al., 2005; Hong and Yaffe, 2006). The concept of co-activator/co-repressor conversion is emerging. Our results in Chapter 2 established that TAZ binds to HDACs. Firstly, this provides an alternative mechanism by which Runx-dependent transcription is regulated. Here, I raise a question: If we ectopically express Runx in a cell line that lacking ALL co-activators of Runx, what would happen? The answer is probably no transcriptional activity since there is little evidence to show that Runx proteins recruit Pol II directly by themselves! So the regulation of Runx proteins is per se the regulation of their co-factors. The regulation of Runx-dependent transcription is mostly, if not all, the regulation co-activation by co-factors. Secondly, that the binding of TAZ to co-repressors raises the possibility that some signaling pathways can convert TAZ from a co-activator to a co-repressor due to different co-factor recruitment. In Chapter 3, we observed that when LATS2 and TAZ were co-transfected, the expression level of TAZ increased dramatically, which seems to be specific to TAZ since we did not observe the same phenomenon with YAP (data not shown). Since LATS2 inactivates TAZ (Chapter 4), the increased level of TAZ upon LATS2 expression indicates that TAZ, in the presence of *hippo*-like signaling pathway,

exerts another unknown function, possibly acting as a repressor. Strong evidence emanates from the co-immunoprecipitation experiment in which the binding affinity of TAZ to HDAC4 increased in the presence of LATS2 (Chapter 4), the three molecules form a trimeric co-repressor complex. But the function of this co-repressor complex is unknown. We predict that this complex could have deacetylase activity. It not only represses transcription but also deacetylases some proteins, although the evidence is currently lacking.

The possible deacetylase activity of TAZ/HDACs/LATS2 complex made me think that this complex could be the deacetylase for histones, particularly H2B. Studies have shown that Ste20-like kinase Mst1 phosphorylates H2B at serine 10, which leads to DNA fragmentation and cell death (Ahn et al., 2006; Cheung et al., 2003). This phosphorylation event is preceded by deacetylation of lysine 11 in H2B. I think it is not unlikely that TAZ/HDACs/LATS2 deacetylases this lysine to facilitate H2B phosphorylation-induced cell death, because we observed that TAZ also mediates apoptosis-like morphology changes in some HEK293 cells when co-expressing LATS2/WW45/Mst1. This is a subset of cells with the rest of them showing nuclear export of TAZ (Chapter 5 and data not shown). So it would be very interesting to 1) confirm that the changes are apoptosis by other examinations 2) if TAZ/HDACs/LATS2 can deacetylase lysine 11 in histone H2B, and 3) in those apoptotic cells, if histone H2B is phosphorylated. These studies could lead to the discovery of a new pathway of apoptosis.

This alternative apoptosis pathway could potentially resolve an additional controversy in this field: the *hippo*-like pathway inactivates YAP to promote apoptosis

while YAP is a co-activator for p73 (Levy et al., 2006; Strano et al., 2005; Strano et al., 2001), a known pro-apoptotic molecule. So the explanation would be that overexpressed or highly activated *hippo*-like pathway converts YAP from a mostly growth promoting molecule into a pro-apoptotic one.

6.3 The interplay between TAZ and LATS2

The aforementioned phenomenon that TAZ protein levels increases upon LATS2 overexpression is just one side of the two. The expression of LATS2 kinase also increased dramatically, and this increase was abolished when the serines 89 and 306 are mutated into alanine (Chapter 4). This strongly suggests that a phosphorylation-dependent positive feedback loop exists between TAZ and LATS2. But the exact "dialogue" between them is still elusive. Accumulating evidence suggests protein modification might be the key. LATS2 is autophosphorylated (McPherson et al., 2004), and the kinase dead mutant shows a much lower expression level than the wild type (data not shown). LATS2 is also acetylated and sumoylated (data not shown). Elucidating the role of each modification of this kinase will greatly benefit our understanding the interplay between TAZ and LATS2.

6.4 The *hippo* pathway and contact inhibition

In a more physiological setting, Chapter 5 demonstrated the mechanism through which TAZ and YAP are regulated by the *hippo*-like pathway, it induces nuclear export of YAP and TAZ when cells are in close contact. The explanation is that LATS2, activated by cell-cell contact, phosphorylates serine 127 in YAP and serine 89 in TAZ. These serines

are the sites for 14-3-3 binding (Kanai et al., 2000). Phosphorylation of these sites leads to stronger 14-3-3 association and nuclear export, thus making the transcription of their target genes come to a halt. Although it is known that Merlin is activated when the cells are confluent (Shaw et al., 1998), little evidence is available to demonstrate the subsequent events following Merlin activation in mammalian cells. The observation we reported in Chapter 4 provides crucial clues regarding the consequences of activation the *hippo*-like pathway. Our study has been confirmed by recent findings in Guan's group (Dong et al., 2007).

The results also add another important mechanism of how monolayered cells control their growth via contact inhibition. YAP is well known for its ability to promote cell proliferation in both *Drosophila* and mammals (Dong et al., 2007; Omerovic et al., 2004; Overholtzer et al., 2006). We performed flow cytometry analysis to assess the role of both wild type and mutant YAP in cell proliferation in mammalian cells. We transiently transfected GFP-YAP wild type and its mutants into HEK293 cells. Using flow cytometry, we fractioned GFP-positive cells into staining buffer containing propidium iodine (PI) and 0.1% triton-X-100. The stained cells were passed through the flow cytometry again and fractions of cells in each cycle were obtained. We found that YAP increased S and G2/M phase fractions (data not shown). Interestingly, the YAP 5SA mutant (S61A, S109A, S127A, S164A, S347A) showed more effect than wild type, suggesting that the mutant lost response to endogenous *hippo*-like pathway. A very similar study on TAZ has shown that the TAZ4SA (S66A, S89A, S117A, S306A) possesses features of an oncogene. It causes cell proliferation, EMT and cell migration in

MCF10A cells (Lei et al., 2008). Whether this YAP 5SA mutant can induce EMT and cell migration remain to be investigated.

When the manuscripts were in preparation, many studies came out, causing a burst of knowledge in the field of cell growth regulation and organ size control. After identifying Yorkie as a key mediator of the *Drosophila hippo* pathway (Huang et al., 2005), Pan's group further showed that phosphorylation of a single serine 168 in Yorkie caused the biological readout of the *hippo* pathway (Dong et al., 2007). *Hippo* phosphorylates this serine and drives Yorkie out of the nucleus, terminating transcription of its target genes. This contrasts with mammalian TAZ and YAP. We found that the regulation of mammalian YAP is mainly through two serine residues, serines 127 and 347 (serines 89 and 306 in TAZ) as discussed previously (summarized in Fig. 1). Our findings have been reproduced by serial of recent studies (Hao et al., 2008; Zhang et al., 2008; Zhao et al., 2007). In the future, more vigorous study on YAP is needed to further elucidate the function and regulation of YAP. These experiments include: 1) using Cre/loxP to generate conditional knockout mice, 2) knock-in mouse model, 3) mass spectrometry (MS) to identify more serines that are phosphorylated by the *hippo* pathway, 4) MS on YAP purified under different conditions such as different cell types and confluence, and 5) these YAP mutants that are unable to be phosphorylated by *hippo* pathway might have become gain-of-function oncogenes. Both cell-based and animal model-based experiments can be done to understand tumorigenesis by these mutant YAP.

References

Ahn, S. H., Diaz, R. L., Grunstein, M., and Allis, C. D. (2006). Histone H2B deacetylation at lysine 11 is required for yeast apoptosis induced by phosphorylation of H2B at serine 10. Mol Cell *24*, 211-220.

Bandura, J. L., and Edgar, B. A. (2008). Yorkie and Scalloped: partners in growth activation. Dev Cell *14*, 315-316.

Barettino, D., Vivanco Ruiz, M. M., and Stunnenberg, H. G. (1994). Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. Embo J *13*, 3039-3049.

Borrow, J., Stanton, V. P., Jr., Andresen, J. M., Becher, R., Behm, F. G., Chaganti, R. S., Civin, C. I., Disteche, C., Dube, I., Frischauf, A. M., *et al.* (1996). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nat Genet *14*, 33-41.

Chakalova, L., Debrand, E., Mitchell, J. A., Osborne, C. S., and Fraser, P. (2005). Replication and transcription: shaping the landscape of the genome. Nat Rev Genet *6*, 669-677.

Cheung, W. L., Ajiro, K., Samejima, K., Kloc, M., Cheung, P., Mizzen, C. A., Beeser, A., Etkin, L. D., Chernoff, J., Earnshaw, W. C., and Allis, C. D. (2003). Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. Cell *113*, 507-517.

Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. Embo J *11*, 1025-1033.

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*, 1120-1133.

Durand, B., Saunders, M., Gaudon, C., Roy, B., Losson, R., and Chambon, P. (1994). Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. Embo J *13*, 5370-5382.

Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997). Nuclear receptor coactivators. Curr Opin Cell Biol *9*, 222-232.

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*, 5496-5509.

Harrington, K. S., Javed, A., Drissi, H., McNeil, S., Lian, J. B., Stein, J. L., Van Wijnen, A. J., Wang, Y. L., and Stein, G. S. (2002). Transcription factors RUNX1/AML1 and RUNX2/Cbfa1 dynamically associate with stationary subnuclear domains. J Cell Sci *115*, 4167-4176.

Hilfiker, A., Hilfiker-Kleiner, D., Pannuti, A., and Lucchesi, J. C. (1997). mof, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in Drosophila. Embo J *16*, 2054-2060.

Hong, J. H., Hwang, E. S., McManus, M. T., Amsterdam, A., Tian, Y., Kalmukova, R., Mueller, E., Benjamin, T., Spiegelman, B. M., Sharp, P. A.*, et al.* (2005). TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. Science *309*, 1074-1078.

Hong, J. H., and Yaffe, M. B. (2006). TAZ: a beta-catenin-like molecule that regulates mesenchymal stem cell differentiation. Cell Cycle *5*, 176-179.

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*, 421-434.

Ito, Y. (2004). Oncogenic potential of the RUNX gene family: 'overview'. Oncogene *23*, 4198-4208.

Ito, Y., Osato, M., and Ito, K. (2003). RUNX and cancer. Ann Acad Med Singapore *32*, S6-7.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science *293*, 1074-1080.

Kanai, F., Marignani, P. A., Sarbassova, D., Yagi, R., Hall, R. A., Donowitz, M., Hisaminato, A., Fujiwara, T., Ito, Y., Cantley, L. C., and Yaffe, M. B. (2000). TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. Embo J *19*, 6778-6791.

Lei, Q. Y., Zhang, H., Zhao, B., Zha, Z. Y., Bai, F., Pei, X. H., Zhao, S., Xiong, Y., and Guan, K. L. (2008). TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. Mol Cell Biol *28*, 2426-2436.

Levy, D., Adamovich, Y., Reuven, N., and Shaul, Y. (2006). The Yes-associated protein 1 stabilizes p73 by preventing Itch-mediated ubiquitination of p73. Cell Death Differ.

McPherson, J. P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak-Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J., *et al.* (2004). Lats2/Kpm is required

for embryonic development, proliferation control and genomic integrity. Embo J *23*, 3677-3688.

Omerovic, J., Puggioni, E. M., Napoletano, S., Visco, V., Fraioli, R., Frati, L., Gulino, A., and Alimandi, M. (2004). Ligand-regulated association of ErbB-4 to the transcriptional co-activator YAP65 controls transcription at the nuclear level. Exp Cell Res *294*, 469-479.

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*, 12405-12410.

Pelletier, N., Champagne, N., Stifani, S., and Yang, X. J. (2002). MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. Oncogene *21*, 2729-2740.

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*, 7757-7764.

Stein, G. S., Lian, J. B., Stein, J. L., van Wijnen, A. J., Choi, J. Y., Pratap, J., and Zaidi, S. K. (2003). Temporal and spatial parameters of skeletal gene expression: targeting RUNX factors and their coregulatory proteins to subnuclear domains. Connect Tissue Res *44 Suppl 1*, 149-153.

Strano, S., Monti, O., Pediconi, N., Baccarini, A., Fontemaggi, G., Lapi, E., Mantovani, F., Damalas, A., Citro, G., Sacchi, A., *et al.* (2005). The transcriptional coactivator Yes-

associated protein drives p73 gene-target specificity in response to DNA Damage. Mol Cell *18*, 447-459.

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*, 15164-15173.

Zaidi, S. K., Sullivan, A. J., Medina, R., Ito, Y., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. (2004). Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. Embo J *23*, 790-799.

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*, 188-192.

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

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L.*, et al*. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev *21*, 2747-2761.

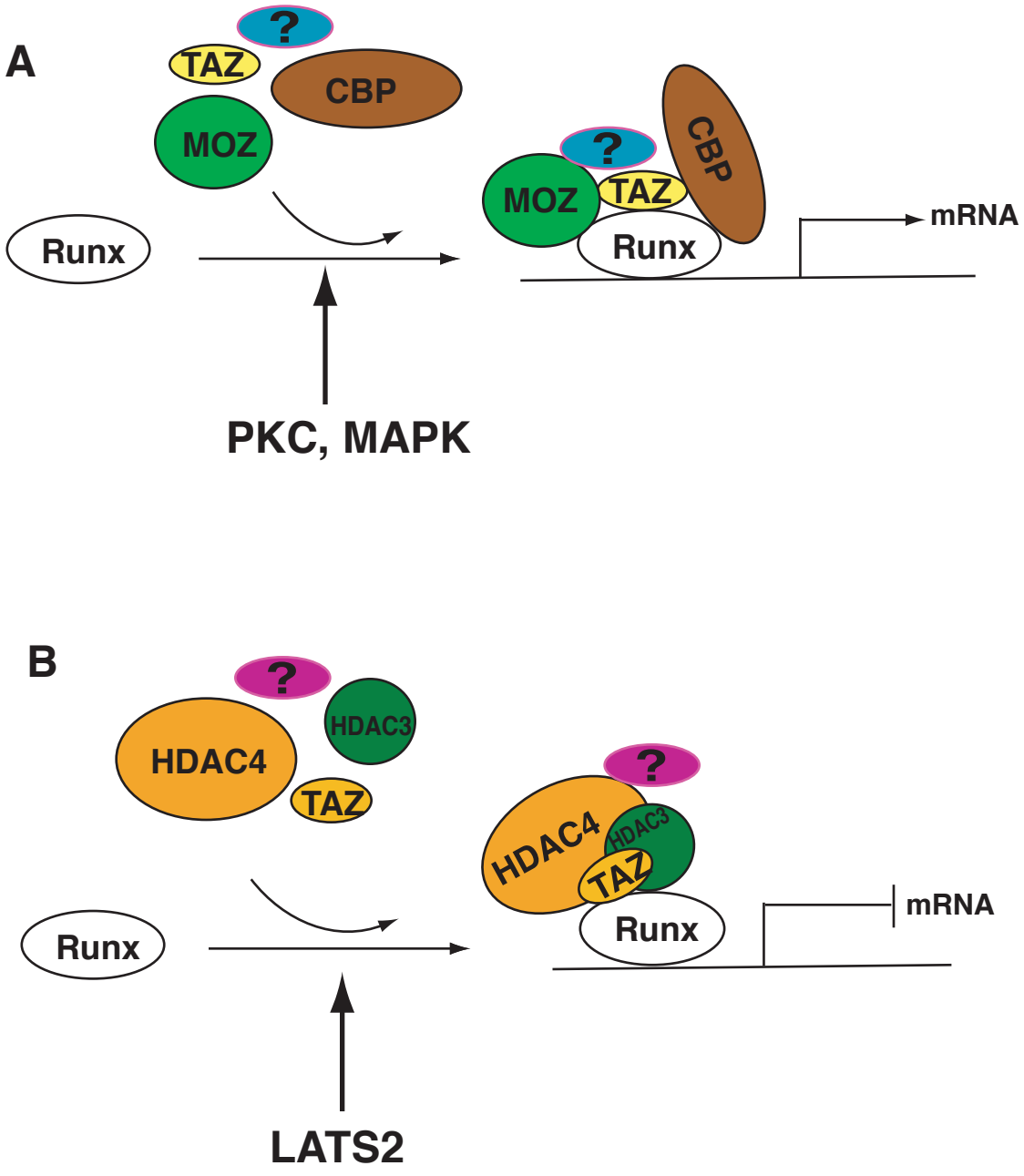
Figure 1

Signaling pathways convert TAZ from a co-activator into a co-repressor

(A) TAZ, MOZ and CBP are known co-activators for Runx proteins. Under the stimulation of the PKC or MAPK pathways, those co-activators form a co-activator complex mediated by TAZ, as TAZ can associate with Runx proteins, MOZ and CBP. TAZ serves as a co-activator.

(B) HDAC3 and HDAC4 are co-repressors for Runx proteins. TAZ physically interacts with both HDAC3 and HDAC4. The affinity between TAZ and HDAC4 is much stronger in the presence of LATS2. LATS2 induced a co-repressor complex mediated by phosphorylated TAZ. TAZ becomes a co-repressor in this context.

Figure 1


A
?
TAZ
CBP
MOZ
Runx
?
CBP
MOZ
TAZ
Runx
mRNA
PKC, MAPK
B
?
HDAC3
HDAC4
TAZ
?
HDAC4
HDAC3
TAZ
Runx
Runx
mRNA
LATS2

Chapter 7

Contribution of Original Research

I obtained novel insights into the molecular basis for the synergistic effect of TAZ and MOZ on Runx-dependent transcription. I further demonstrated that the synergy is dependent on the PPXY motif in Runx proteins. I also confirmed the roles of PKC and MAPK pathways in the up-regulation of Runx-dependent transcription. Lastly, in a more physiological relevant context, the ELISA study provides evidence suggesting that TAZ is a signal-responsive co-factor for Runx proteins.

I examined the roles of different groups of HDACs in the regulation of Runx-dependent transcription. First, I showed that HDACs regulate the transcriptional activities of Runx proteins through their co-factors. I also showed, for the first time, that TAZ is acetylated and that this modification is important in the regulation of the function of TAZ. I also provided evidence that TAZ can be a transcriptional co-repressor by recruiting HDACs.

I provided the first thorough demonstration of the mode of action of the hippo-like pathway in mammalian cells. We found the long sought kinases for TAZ and YAP. I identified the phosphorylation site of LATS2 on TAZ and YAP. I provided a fairly complete picture of the consequences of this phosphorylation event. Phosphorylation of TAZ and YAP leads to their nuclear export and transcriptional inactivation. In addition, I showed the link between LATS2 and HDACs, providing opportunities for probing more deeply into the consequences of phosphorylation.

By bringing in a more physiological setting to look into the effect of cell-cell contact on the regulation of the function of TAZ and YAP, I found that cell-cell contact is a potent input to activate the hippo-like pathway, which results in nuclear export of TAZ

and YAP. I showed that cell-cell contact results in increased phosphorylation of serine 127 in YAP, thereby leading to increased 14-3-3 binding.

Although most of the data has not been published yet, the results generated in the thesis work have paved the way for the ongoing research in Dr. Yang's laboratory. The thesis work was finished in Aug. 2007. The field turned out to be highly competitive. The findings in Chapter 4 and 5 are novel, and are consistent with several independent reports that have been published from several other laboratories afterwards (Bandura and Edgar, 2008; Dong et al., 2007; Hao et al., 2008; Zeng and Hong, 2008; Zhang et al., 2008; Zhao et al., 2007).

References

Bandura, J. L., and Edgar, B. A. (2008). Yorkie and Scalloped: partners in growth activation. Dev Cell *14*, 315-316.

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*, 1120-1133.

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*, 5496-5509.

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*, 188-192.

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

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L.*, et al*. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev *21*, 2747-2761.